Wiki Lab Protocols:

Resuspension of artemisinic alcohol dehydrogenase (ADH1), artemisinic aldehyde dehydrogenase (ALDH1) and artemisinic aldehyde ∆11 (13) reductase (DBR2) synthesised DNA from IDT:

Objective: To resuspend the dry DNA.

Requirements:

- Synthesised DNA
- 10µL of TE Buffer per resuspension
- P20 calibrated pipette
- Pipette tips
- Micro-centrifuge

Procedure:

- Add 10µL of TE buffer to each of the tubes containing dry DNA (each tube contained 1000ng of dry DNA).
- Shake tubes to resuspend DNA.
- Pulse tubes in microcentrifuge.

Results:
Synthesised DNA was made to a concentration of 100 ng/µL.

Digestion and Ligation of Synthesised DNA:

Objective: To ligate the linearized synthesised DNA to a chloramphenicol resistant plasmid backbone and also to ligate the synthesized DNA Ribosome Binding Site (RBS) and a kanamycin backbone.

Requirements:

- NEBuffer 2.1
- Eco-RI HF®
- PstI
- SpeI
- XbaI
- dH2O
- Pipettes, pipette tips
- Water bath and/or heat block/thermocycler
- eppendorf tubes
- permanent marker
- T4 DNA ligase reaction buffer
- T4 DNA ligase
- ice box

Procedure (based on iGEM protocol http://parts.igem.org/Help:Protocols/3A_Assembly)

- Calculate the required amount of enzyme master mixes.

Prepare Enzyme master mix for plasmid backbone by adding the following to an eppendorf tube:
- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL PstI
- 18.5µL dH2O

Prepare Enzyme master mix for Part A by adding the following to an eppendorf tube:
- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL SpeI
- 18.5µL dH2O

Prepare Enzyme master mix for Part B by adding the following to an eppendorf tube:
- 5.5µL NEBuffer 2.1
- 0.5µL PstI
- 0.5µL XbaI
- 18.5µL dH2O

Digest synthesised ADH1 (conc. 100 ng/µL) for ligation to chloramphenicol backbone by adding the following to a labelled eppendorf tube:
- 1µL ADH1
- 3µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest synthesised ALDH1 (conc. 100 ng/µL) for ligation to chloramphenicol backbone by adding the following to a labelled eppendorf tube:
- 1µL ALDH1
- 3µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest synthesised DBR2 (conc. 100 ng/µL) for ligation to chloramphenicol backbone by adding the following to a labelled eppendorf tube:
- 1µL DBR2
- 3µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest RFP chloramphenicol (BBa_J04450) by adding the following to a labelled eppendorf tube:
- 100ng RFP chloramphenicol
- 4µL enzyme master mix for plasmid backbone

Digest RFP Kanamycin (BBa_J04450) by adding the following to a labelled eppendorf tube:
- 100ng RFP Kanamycin
- 4µL enzyme master mix for plasmid backbone

Digest RBS Strong (BBa_B0034) by adding the following to a labelled eppendorf tube:

- 100ng RBS Strong
- 4μL enzyme master mix for part A

Digest RBS Weak (B0032) by adding the following to a labelled eppendorf tube:
- 100ng RBS Weak
- 4μL enzyme master mix for part A

Digest synthesised ADH1 (conc. 100 ng/μL) for ligation to RBS and kanamycin backbone by adding the following to a labelled eppendorf tube:
- 1μL ADH1
- 3μL dH2O
- 4μL enzyme master mix for part B

Digest synthesised ALDH1 (conc. 100 ng/μL) for ligation to RBS and kanamycin backbone by adding the following to a labelled eppendorf tube:
- 1μL ALDH1
- 3μL dH2O
- 4μL enzyme master mix for part B

Digest synthesised DBR2 (conc. 100 ng/μL) for ligation to RBS and kanamycin backbone by adding the following to a labelled eppendorf tube:
- 1μL DBR2
- 3μL dH2O
- 4μL enzyme master mix for part B

Incubate the digestions at 37^{o}C for 30 minutes. Heat-kill at 80^{o}C for 20 minutes.

Ligation:

Ligate the digested synthesised DNA to the chloramphenicol backbone by adding the following to a new eppendorf tube:
- 2μL digested RFP chloramphenicol
- 2μL of the appropriate digested synthesised DNA
- 1μL T4 DNA ligase reaction buffer
- 0.5μL T4 DNA ligase
- 4.5μL dH2O

Ligate the digested synthesised DNA to the chosen RBS and kanamycin backbone by adding the following to a new eppendorf tube:
- 2μL digested RFP kanamycin
- 2μL digested appropriate RBS (ligate strong to all three synthesised genes, ligate weak to ADH1 and ALDH1)
- 2μL digested appropriate gene
- 1μL T4 DNA ligase reaction buffer
- 0.5μL T4 DNA ligase
- 2.5μL dH2O

Incubate the ligations at 16^{o}C for 30 minutes. Heat-kill at 80^{o}C for 20 minutes.

Transformation: (based on NEB High Efficiency Transformation protocol)

Objective: To transform XL1-blue cells with the ligation products.

Requirements:

- 50µL of competent cells per transformation
- Ligation product
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42^{o}C
- Ice box
- Incubator
- 950µL of SOB medium per transformation
- eppendorf tubes
- bunsen burner
- Glass spreader
- Ethanol

- Pre-chill eppendorf tubes on ice.
- Thaw competent cells on ice.
- Add 50µL of competent cells to each of the pre-chilled transformation tubes.
- Add 2µL of ligation product to its appropriate transformation tube.
- Carefully flick the transformation tubes 4-5 times to mix the cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes.
- Heat-shock at 42^{o}C in the heat-block for exactly 30 seconds.
- Place the transformations on ice for 5 minutes.
- Pipette 950µL of SOB medium into each transformation tube.
- Incubate the transformations at 37^{o}C for an hour with shaking.
- Warm selection plates to 37^{o}C
- Spin the cells at 5g for 2 minutes.
- Remove 800µL of the supernatant from each tube and discard.
- Aspirate a number of times to resuspend the pellet before plating what's left in the transformation tube.
- Pass the glass spreader through the bunsen burner's flame before spreading.
- Cool the glass spreader on part of the plate that does not contain cells before spreading the cells on the plate.
- Incubate plates overnight at 37^{o}C

Results:

Very few colonies grew. It was planned to use PCR to amplify the synthesised DNA and to design primers that would extend the overhangs to improve the action of restriction enzymes.

Polymerase Chain Reaction (PCR) amplification of synthesised DNA received from IDT:

Objective: To amplify synthesised DNA using primers designed to extend overhangs in order to improve action of restriction enzymes.

Requirements:
Q5® High-Fidelity 2X Master Mix
Forward and Reverse Primers (primers came dry from Sigma)
TE Buffer / Nuclease free H2O
Ice box
0.2 mL eppendorf tubes
Thermocycler
Pipettes, pipette tips

Procedure: (Based on NEB Protocol for Q5® High-Fidelity 2X Master Mix)

The concentration of BioBrick primer R is 99.5 nm/ml and the concentration of primer BioBrick F is 91.1 nm/ml.

- Add 995µL of TE buffer to the reverse primer BioBrick R and 911µL of TE buffer to the forward primer BioBrick F.
- Vortex the tubes and centrifuge quickly.
- Add 10µL of each primer to a new eppendorf tube. Both primers should then have a concentration of 50µM.
- Add 9 parts TE/water to 1 part primer. ie. add 180µL TE a concentration of 5µM of each primer.
- Centrifuge the IDT synthesised genes quickly.
- Add TE buffer up to 0.5ml eppendorfs.
- Add the IDT genes, ADH1, ALDH1 and DBR2, to the eppendorfs containing the TE buffer separately. These are to become the template DNA.
- 9 tubes are to undergo the PCR. The 9 tubes are labelled with the name of the gene to be added, 3 ADH1, 3 ALDH1 and 3 DBR2. Fill three of the tubes with triple the necessary volume. ie. 150µL. Distribute the 150µL to the other 6 tubes. Each tube should contain: 25µL of Q5 High-Fidelity master mix, 15µL of primer mix, 1µL of the appropriate primer and 19.6µL of water.
- Place the samples into the thermocycler and set the appropriate program
- Three of the tubes are set to have an annealing temperature at 60^{o}C, three at 65^{o}C and three at 70^{o}C.

Results:

The three PCR products which had an annealing temperature of 70^{o}C were found to have the highest concentration of DNA.

Digestion and Ligation of PCR products 5th August:

Objective: To ligate the PCR products to a Ribosome Binding Site (RBS) and a kanamycin backbone.

Requirements:

- Purified DNA
- NEBuffer 2.1
- Eco-RI HF®
- PstI
- SpeI
- XbaI
- dH2O
- Pipettes, pipette tips
- Water bath and/or heat block/thermocycler
- eppendorf tubes
- permanent marker
- T4 DNA ligase reaction buffer
- T4 DNA ligase
- ice box

Procedure (based on iGEM protocol http://parts.igem.org/Help:Protocols/3A_Assembly)

- Calculate the required amount of enzyme master mixes.

Prepare Enzyme master mix for plasmid backbone by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL PstI
- 18.5µL dH2O

Prepare Enzyme master mix for Part A by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL SpeI
- 18.5µL dH2O

Prepare Enzyme master mix for Part B by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL PstI
- 0.5µL XbaI
- 18.5µL dH2O

Digest RFP kan plasmid backbone (conc. 114.8ng/µL) adding the following into two eppendorf tube:

- 0.87µl plasmid backbone
- 3.12 µL dH2O
- 4 µL Enzyme master mix for plasmid backbone

Digest RBS strong (conc. 143.9ng/µL) part B0034 by adding the following into two eppendorf tubes:

- 0.69µL RBS
- 3.3µL dH2O

- 4μL Enzyme master mix for Part A

Digest RBS Amp weak (conc. 99.5ng/μL) part B0032 by adding the following into two eppendorf tubes:
- 1μL RBS
- 2.99μL dH2O
- 4μL Enzyme master mix for Part A

Digest ALDH1 PCR product (conc.97.2ng/μL) by adding the following to two eppendorf tubes:
- 1.02μL ALDH1
- 2.97μL dH2O
- 4μL Enzyme master mix for Part B

Digest ADH1 PCR product (conc. 89ng/μL) by adding the following to two eppendorf tubes:
- 1.12μL ADH1
- 2.88μL dH2O
- 4μL Enzyme master mix for Part B

Digest DBR2 PCR product (conc. 111.4ng/μL) by adding the following to a single eppendorf tube:
- 0.9μL DBR2
- 3.1μL dH2O
- 4μL Enzyme master mix for Part B

Incubate at 37^{o}C for 30 minutes in a water bath then heat killed at 80^{o}C for 20 minutes in a heat block.

Ligation:
- Add 2μL of the digested plasmid backbone, 3 μL of digested RBS strong and 3μL of digested ALDH1 to one eppendorf tube.
- Add 2μL of the digested plasmid backbone, 3 μL of digested RBS weak and 3μL of digested ALDH1 to one eppendorf.
- Add 2μL of the digested plasmid backbone, 3 μL of digested RBS strong and 3μL of digested ADH1 to one eppendorf.
- Add 2μL of the digested plasmid backbone, 3 μL of digested RBS weak and 3μL of digested ADH1 to one eppendorf.
- Add 2μL of the digested plasmid backbone, 3 μL of digested RBS strong and 3μL of digested DBR2 to another eppendorf.
- Add 1μL of T4 DNA ligase buffer to each eppendorf tube.
- Add 0.5μL of T4 DNA ligase to each tube.
- Add 0.5μL of dH2O to each tube.

Incubate at 16^{o}C celsius for 30 minutes in a water bath before being heat killed at 80^{o}C for 20 minutes in a heat block.

Transformation: (based on iGEM protocol http://parts.igem.org/Help:Protocols/Transformation)

Objective: To transform the ligation products into DH5alpha cells.

Requirements:

- 50µL of competent cells per transformation
- purified DNA
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42^{o}C
- Ice box
- Incubator
- 950µL of SOB medium per transformation
- eppendorf tubes
- bunsen burner
- Glass spreader
- Ethanol

- Thaw competent DH5 alpha cells on ice and pre-chill eppendorfs on ice.
- Add 50µL of cells to 5 eppendorf tubes.
- Add 1µL of the appropriate ligation to its corresponding tube.
- Chill the transformations on ice for 30 minutes.
- Heat-shock the tubes at 42^{o}C in a heat block for 60 seconds.
- Incubate the tubes on ice for 5 minutes.
- Add 200µL of SOC medium to each tube.
- Incubate the tubes at 37^{o}C for 2 hours with shaking.
- Spin the tubes at 5g for 2 minutes.
- Remove 150µL of the supernatant.
- Resuspend the cells in the remaining supernatant by vortexing for about 3-4 minutes; and aspirate using a pipette.
- Transfer 100µL of the transformation to the appropriately labelled kanamycin plate.
- Leave the plates upside in an incubator for about 20 hours.

Results:

Sequencing results received on the 12th August showed that RBS strong + DBR2 sample A1 had been successfully ligated but the other samples that were sequenced hadn't been successfully constructed.

See our timeline for a general overview of the protocols carried out between 5th August - 31st August. On 31st August, we received sequencing results which showed that none of our final constructs contained the DBR2 gene. We decided to repeat the 3A assembly techniques and to carry out PCR on miniprep samples to confirm part size as we progressed.

Preparation of B569 Cell Broth:

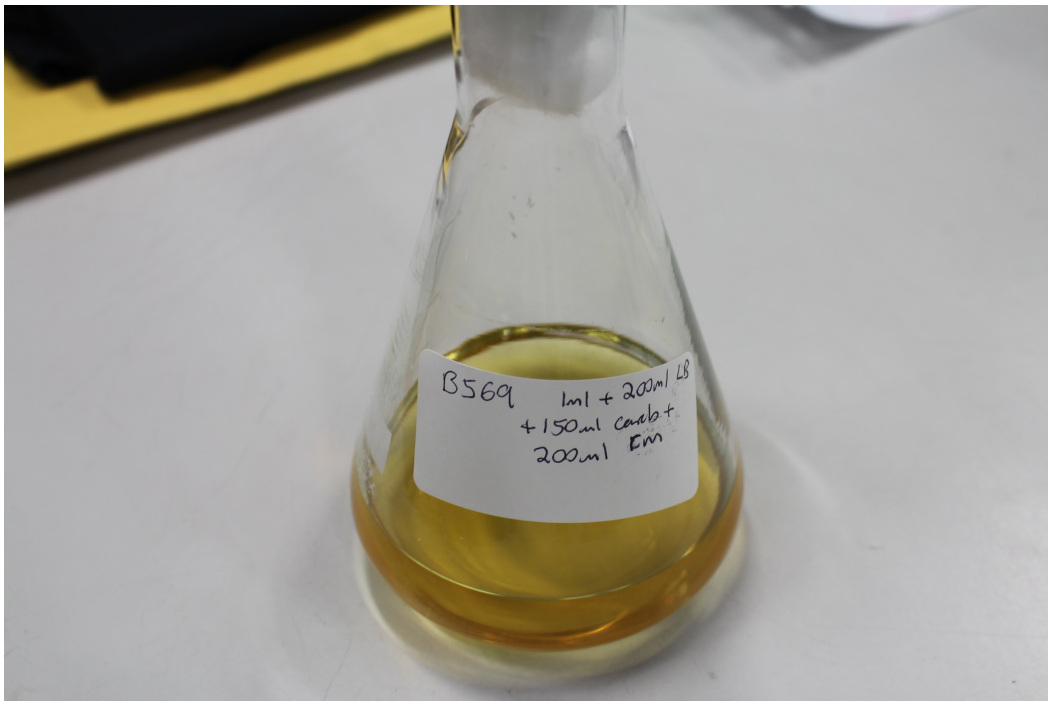
Objective: To make a stock of B569 cells. These cells were engineered by Amyris to produce artemisinic acid. We hoped that by transforming the plasmid we would construct into these cells we would be able to increase yields of artemisinic acid / dihydroartemisinic acid.

Requirements:

- Glycerol stock of B569 cells
- 200mL LB medium
- 200µL chloramphenicol (1000x)
- 150µL carbenicillin (1000x)
- Conical flask
- Incubator with shaker

Procedure:

- Retrieve B569 glycerol stock from the -80°C freezer.
- Add the following to a conical flask working beside the bunsen:
  - ❏ 200mL of LB medium
  - ❏ 200µL of chloramphenicol
  - ❏ 150µL of carbenicillin
  - ❏ 1mL of B569 cells
- Stuff top of conical flask with cotton wool.
- Store in incubator for 14 hours at 33.6°C with shaking.



Preparation of Competent B569 cells 19th August: (Based on iGEM protocol http://parts.igem.org/Help:Protocols/Competent_Cells)

Objective: To prepare competent B569 cells that will be able to be transformed with plasmid DNA.

Requirements:

- 2.35g $CaCl_2$
- 0.2g KOAc
- 0.8g $MnCl_2$
- 0.4g $MgCl_2$
- 180mL dH2O
- 20mL Glycerol
- B569 broth
- Falcon tubes
- Centrifuge
- Ice box
- Pasteur Pipettes
- Vortex

Prepare CCMB80 buffer by combining the following in a glass bottle:

- 2.35g $CaCl_2$
- 0.2g KOAc
- 0.8g $MnCl_2$
- 0.4g $MgCl_2$
- 180mL dH2O to dissolve the salts
- 20mL Glycerol

Store solution in the fridge. Put on ice before use.

- Store B569 broth on ice. Use the spectrometer to test optical density (option wasn't available at time protocol was carried out).
- Pre-chill 4 50mL falcon tubes on ice (didn't have access to flat-bottom centrifuge bottles).
- Divide the B569 broth between the 4 tubes.
- Centrifuge the tubes at 3000g for 10 minutes.
- Very large cell pellets will form (if spectrometer hasn't been used to monitor cell density) and the pellets will fill the entire pointed bottom of each tube (if falcon tubes have been used instead of flat-bottom centrifuge bottles).
- Decant the supernatant.
- Pipette 20mL of ice-cold CCMB80 buffer onto the wall of one of the tubes.
- Store other cell pellets in fridge.
- Vortex the tube containing the buffer until the pellet is resuspended.
- Add 10mL of this solution to a new tube.
- Add 30mL of ice-cold CCMB80 buffer to each of the two tubes.
- Incubate the two tubes on ice for 30 minutes.
- Centrifuge the tubes at 3000g for 10 minutes.
- Decant the supernatant.
- Attempt to resuspend pellets by adding 5mL of ice-cold CCMB80 buffer to each tube.
- If pellet can't be resuspended, vortex the tube until most of the pellet is resuspended.
- Recombine the suspension in a new tube.
- Keep the tube on ice for 20 minutes.
- Pre-chill 2mL eppendorf tubes on ice.
- Make aliquots of 500µL
- Store aliquots at -80^{o}C.

- Carry out a competency test.

Digestion and ligation 1st September:

Objective: The aim of the procedure is to ligate the weak RBS (ie. BBa_B0032) with ALDH1 and a kanamycin backbone. Use resuspended synthesised IDT DNA specifically designed with longer overhangs and His-tags.

Requirements:
- NEBuffer 2.1
- Eco-RI HF®
- PstI
- SpeI
- XbaI
- dH2O
- Pipettes, pipette tips
- Water bath and/or heat block/thermocycler
- eppendorf tubes
- permanent marker
- T4 DNA ligase reaction buffer
- T4 DNA ligase
- ice box

Procedure (based on iGEM protocol http://parts.igem.org/Help:Protocols/3A_Assembly)

- Carry out procedure in triplicate.
- Calculate the required amount of enzyme master mixes.

Add the following to a tube labelled Enzyme master mix plasmid backbone:
- 5.5µL NEBuffer 2.1
- 0.5µL EcoRI
- 0.5µL PstI
- 18.5µL dH2O

Add the following to a tube labelled Enzyme master mix for Part A:
- 5.5µL NEBuffer 2.1
- 0.5µL EcoRI
- 0.5µL SpeI
- 18.5µL dH2O

Add the following to two tubes labelled Enzyme master mix for Part B:
- 5.5µL NEBuffer 2.1
- 0.5µL XbaI
- 0.5µL PstI
- 18.5µL dH2O

Digest RFP Kanamycin (conc.167.5ng/µL) by adding the following to a labelled eppendorf tube:

- 0.6μL RFP Kan
- 3.4μL dH2O
- 4μL Enzyme master mix for plasmid backbone

Digest RBS weak (conc. 99.5ng/μL) by adding the following to a labelled eppendorf tube:
- 1μL RBS weak
- 3μL dH2O
- 4μL Enzyme master mix for part A

Digest ALDH1 (with His Tags)(directly from resuspended IDT synthesised DNA) (conc. 100ng/μL) by adding the following to a labelled eppendorf tube:
- 1μL ALDH1
- 3μL dH2O
- 4μL Enzyme master mix for part B

Incubate the digestions at 37^{o}C for 30 minutes. Move the digestions to a heat-block set at 80^{o}C for 20 minutes.

Ligation:

Add the following to a labelled eppendorf tube to ligate together RBS weak and ALDH1:
- 1μL digested RFP Kanamycin
- 3μL digested RBS Weak
- 3μL digested ALDH1 with His-Tags
- 1μL T4 DNA Ligase Reaction Buffer
- 0.5μL T4 DNA Ligase
- 1.5μL dH2O

Incubate the ligations at 16^{o}C for 30 minutes and then heat-killed at 80^{o}C for 20 minutes.

Transformation 1st September: (based on NEB High Efficiency Transformation protocol)

Objective: To transform the ligation product (RBS weak + ALDH1) into DH5alpha cells.

Requirements:
- 50μL of competent cells per transformation
- Ligation product
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42^{o}C
- Ice box
- Incubator
- 950μL of SOB medium per transformation
- eppendorf tubes
- bunsen burner
- Glass spreader
- Ethanol

- Add 50µL of competent DH5alpha cells to two pre-chilled eppendorf tubes.
- Add 2µL of the ligation product to the two tubes.
- Keep the transformations on ice for 30 minutes.
- Heat-shock the tubes at 42^{o}C for 30 seconds.
- Store the transformations on ice for 5 minutes.
- Add 950µL of SOB medium to each tube.
- Incubate the transformations for 1 hour with shaking at 37^{o}C.
- Plate 200µl of one of the transformations straight onto a kan plate and spread using a glass spreader.
- Spin the rest of the transformations at 5g for 2 minutes before removing 800µl of the supernatant.
- Resuspend the pellet in what's left by aspirating using the pipette.
- Plate the transformations.
- Incubate the plates overnight at 37^{o}C.

Results:
The following day there were colonies on all of the plates. In comparison with the other plates, there were less colonies on the plate in which the transformation tube hadn't been centrifuged and the supernatant removed.

Preparation of 400mL of LB medium 2nd September:

Requirements:

- Balance
- Spatula
- Weighing Boat
- 500mL Glass Bottle
- 4g Sodium Chloride
- 4g Tryptone Enzymatic Digest from Casein
- 2g Yeast Extract
- 400mL dH2O
- Autoclave Tape
- Permanent Marker
- Aluminium foil
- Access to autoclave

Procedure:

Add the following to a labelled glass bottle:
- 4g Sodium Chloride
- 4g Tryptone Enzymatic Digest from Casein
- 2g Yeast Extract

- Dissolve in 350ml dH2O
- Make up solution to 400mL

- Loosen the cap of the glass bottle
- Cover the cap in aluminium foil
- Seal foil using autoclave tape
- Autoclave at 120^{o}C for 20 minutes

Preparation of 400mL of LB Agar:

Objective: To prepare plates for selection.

Requirements:
- 4g NaCl
- 4g Tryptone Enzymatic Digest from Casein
- 2g Yeast Extract
- 6g Agar
- Glass bottle
- 400mL dH2O
- Spatula
- Balance
- Weighing boat
- Aluminium foil
- Autoclave tape
- Permanent marker
- Petri dishes
- Access to autoclave
- Flow cabinet / bunsen burner
- 1000x antibiotic solution
- P1000 pipette, pipette tips
- Parafilm

Procedure:

Add the following to a glass bottle:

- 4g NaCl
- 4g Tryptone Enzymatic Digest from Casein
- 2g Yeast Extract
- ~350mL of water

- Dissolve and make up to 400mL
- Add 6g Agar
- Loosen the cap of the glass bottle
- Cover the cap in aluminium foil
- Seal foil using autoclave tape
- Autoclave at 120^{o}C for 20 minutes
- Allow to cool until bottle is touchable
- In the flow cabinet: Add 400µL of 1000x antibiotic.
- Swirl the bottle to mix the antibiotic
- Pour plates
- Place the lid of the plate over half of the plate and leave for ~20 minutes to solidify

- Cover the plates with their lids
- Label plates and seal vents with parafilm
- Store plates at 4^{o}C

Preparation of Broths 2nd September:

Objective: To prepare broths from all of the transformation plates made the previous day (ie. RBS weak + ALDH1) after 18 hours of incubation at 37^{o}C.

Requirements:
- 5mL LB medium per broth
- Appropriate antibiotic
- Tubes
- Incubator with shaker
- Transformation plates
- Wire loop
- Bunsen burner
- Permanent marker

Procedure: (perform beside a lighted bunsen burner)
- Add 5mL of LB medium to a falcon tube.
- Add 5µL of the appropriate 1000x antibiotic (kanamycin).
- Use a sterile loop to scoop up a white colony from each plate.
- Inoculate the LB medium in the falcon tube by placing the loop into the tube and stirring.
- Sterilise the loop by putting it into the bunsen burner's flame before repeating the process for the desired amount of broths.
- Incubate broths overnight at 37^{o}C with shaking.

Miniprep 3rd September:

(Based on Qiagen protocol)

Objective: To obtain purified RBS weak +ALDH1 DNA.

Requirements:
- Qiagen QIAprep Spin Miniprep Kit
- Cell Broths
- eppendorf tubes
- P200, P1000 calibrated pipettes
- Pipette tips
- Nanodrop
- Micro-Centrifuge

Procedure:
- Add 1mL of broth to eppendorf tube and centrifuge at 8000rpm for 3 minutes. Discard the supernatant.

- Resuspend the cells in 250µL of Buffer P1. Add RNase A and LyseBlue to this buffer before use.
- Add 250µL of Buffer P2 and invert the tubes 4-6 times. The cell suspension should turn blue.
- Add 350µL Buffer N3 after 5 minutes. Invert the tubes 4-6 times until the solution is no longer blue. The solution should become cloudy.
- Centrifuge the tubes at 13,000rpm for 10 minutes. A white pellet should form.
- Add 800µL of the supernatant to the QIAprep 2.0 spin column by pipetting. Do not disturb the white pellet.
- Centrifuge the columns for 60 seconds. Discard the flow-through.
- Wash the QIAprep 2.0 spin column with 750µL of Buffer PE and centrifuge for 60 seconds.
- Discard the flow-through and centrifuge the columns again for 1 minute to remove residual wash buffer.
- Place the Qiaprep 2.0 spin column in a 2mL eppendorf tube. To elute the DNA, add 50µL of Buffer EB to the centre of each column. Allow to columns to stand for one minute then centrifuge for one minute.
- Check the concentrations of the minipreps using the nanodrop.

Preparation of a 2% Agarose Gel 3rd September:

Objective: To prepare a 2% Agarose Gel to run PCR products ~ 200bp to confirm part size.

Requirements:
- 1.5g Agarose
- ~400mL 1x TBE buffer
- 7.5µL GelRed
- Glass Bottle
- Gel Tray
- Electrodes
- Gel Comb
- P20 Pipette
- Pipette tips
- 25mL Pasteur Pipette
- Microwave

Procedure:

- Add 1.5g of agarose to a glass bottle.
- Add 75mL of 1x TBE Buffer.
- Swirl the solution and microwave until the agarose fully dissolves. Make sure to loosen the top of the glass bottle before placing it in the microwave. Be careful when handling the bottle. The solution should resemble water when the agarose is fully dissolved.
- When the solution is cool enough to touch, add 7.5µL of GelRed Nucleic Acid Gel Stain. Make sure to protect the GelRed from light when putting it back into storage.
- Swirl the glass bottle.
- Pour the gel into the gel tray.
- Remove bubbles using a pipette tip.

- Add the combs and leave the gel to solidify for a few hours.

PCR 3rd September:

Objective: To determine if purified plasmid DNA contains the desired part. ie. Weak RBS + ALDH1.

Requirements:
- Q5® High-Fidelity 2X Master Mix
- Forward and Reverse Primers
- TE Buffer / Nuclease free H2O
- Ice box
- 0.2 mL eppendorf tubes
- thermocycler
- Pipettes, pipette tips

Procedure (Based on NEB Protocol for Q5® High-Fidelity 2X Master Mix)

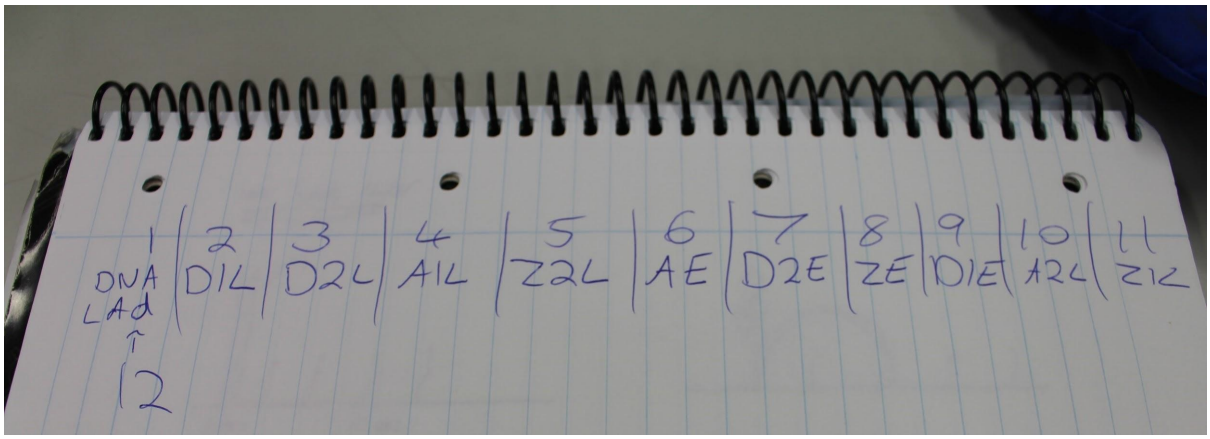
- Design forward PCR primer to anneal to Weak Ribosome Binding site.
- Design reverse primer to anneal to ALDH1 gene.
- Make a master mix for the primer pair. Ten samples of hypothetical RBS + ALDH1 were to be amplified. Create master mix for twelve samples to allow for pipetting error. Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:
- 150µL Q5® High-Fidelity 2X Master Mix
- 15µL of 10µM (add 10µL of 100µM forward primer to 90µL of TE Buffer in a separate tube) forward primer
- 15µL of 10µM reverse primer
- 96µL TE Buffer
- Check the tube size that the thermocycler can hold. Add 23µL of the master mix to ten new 0.2mL tubes.
- Label the tubes according to the template DNA to be added.
- Add 2µL of template DNA to the corresponding labelled 0.2mL tube that already contains the master mix. In this case the PCR was carried out on the following Weak RBS + ALDH1 samples: Dáire Late One; Dáire Late Two; Arnas Late One, Zaneta Late Two; Arnas Early; Dáire Early Two; Zaneta Early; Dáire Early One; Arnas Late Two and Zaneta Late One.
- Gently mix the reaction. Collect all liquid in the bottom of the tubes by a quick spin in the centrifuge if necessary.
- Transfer the PCR tubes to the thermocycler.
- Use the NEB Tm Calculator to determine the annealing temperature.
- Set the program on the thermocycler:
- ❏ Initial Denaturation: 98^{o}C for 30 seconds
- ❏ 32 cycles: 98^{o}C for 5-10 seconds; 62^{o}C for 10-30 seconds; 72^{p}C for 20-30 seconds.
- ❏ Final Extension: 72^{o}C for 2 minutes.

Protocol: running PCR products on a 2% agarose gel 3rd September:

Objective:
To determine if weak Ribosome Binding Site had successfully ligated to the AlDH1 Gene.

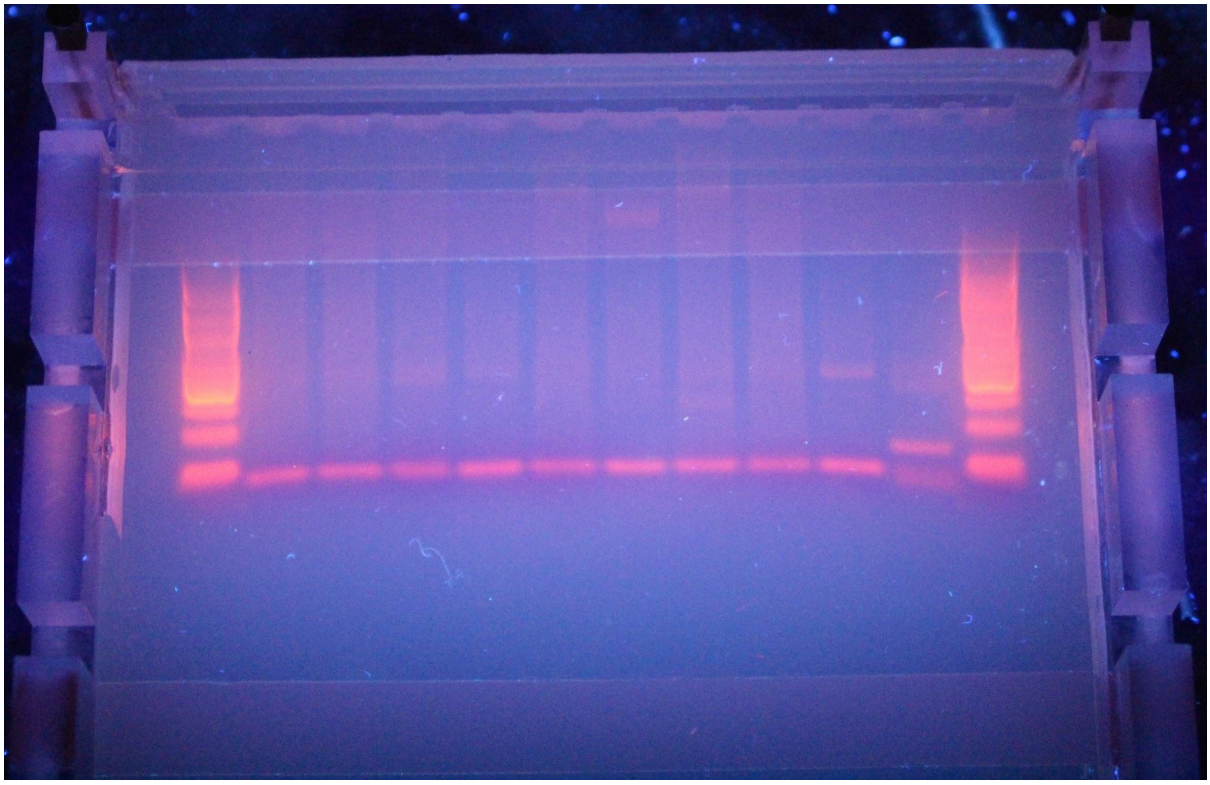
Requirements:
- 2% agarose gel
- PCR products
- 1x TBE buffer
- NEB Gel Loading Dye, Purple 6x
- NEB 100bp DNA ladder
- P20 calibrated pipette, pipette tips
- Eppendorf tubes
- Electrodes
- Gel cover
- Power source
- UV light source

Procedure:
- Prepare a 2% agarose gel.
- When gel has set, position it correctly in the gel tray and remove the comb.
- Transfer 15µL of each PCR product into a new labelled eppendorf tube.
- Add 3µL of NEB Gel Loading Dye, Purple 6x to each sample. Add the appropriate volume of NEB Gel Loading Dye, Purple 6x to a sample of NEB 100bp DNA ladder also.
- Submerge gel in 1x TBE buffer.
- Create a plan as to which lane each sample will run on as shown below:



- Add 18µL of each dyed sample to the appropriate lane using a pipette.
- Place the cover on the gel.
- Attach the electrodes, remembering that the DNA will run towards the red (positive) electrode.
- Run the gel at a low voltage (60-80V) for a few minutes.
- Look at the gel under UV light. Compare sample sizes to DNA Ladder to determine if ligation was carried out successfully.

Results and Conclusions:



Nine of the ten samples showed bands of the expected length. As the forward primer was designed to anneal to the weak ribosome binding site, the evidence from the gel suggested that in nine of the ten samples the PCR had been carried out successfully; and so the RBS was present in the samples. This infers that in nine of the ten samples the previous ligation of the weak RBS to ALDH1 had been successful.

Digestion and Ligation 3rd September:

Objective:
To ligate a terminator (BBa_B0015) to RBS + ALDH1 samples and to ligate promoters D2 (from Device 2 in the interlab study ie. BBa_K823008) and PTAC (BBa_K864400) to RBS + DBR2 A1 sample that had previously been sequenced confirmed.

Requirements:

- NEBuffer 2.1
- Eco-RI HF®
- PstI
- SpeI
- XbaI
- dH2O
- Pipettes, pipette tips
- Water bath and/or heat block/thermocycler
- eppendorf tubes
- permanent marker
- T4 DNA ligase reaction buffer

- T4 DNA ligase
- ice box

Procedure (based on iGEM protocol http://parts.igem.org/Help:Protocols/3A_Assembly)

- Calculate the required amount of enzyme master mixes.

Prepare Enzyme master mix for plasmid backbone by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL PstI
- 18.5µL dH2O

Prepare Enzyme master mix for Part A by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL SpeI
- 18.5µL dH2O

Prepare Enzyme master mix for Part B by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL PstI
- 0.5µL XbaI
- 18.5µL dH2O

Digestion:

Digest RFP Ampicillin (BBa_J04450) (conc. 159.7ng/µL) by adding the following to a labelled eppendorf tube:

- 0.63µL RFP Amp
- 2.52µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest RBS + ALDH1 ZL1 (conc. 54 ng/μL) by adding the following to a labelled eppendorf tube:

- 1.85μL RBS + ALDH1 ZL1
- 2.15μL dH2O
- 4μL enzyme master mix part A

Digest RBS + ALDH1 ZE (conc. 100ng/μL) by adding the following to a labelled eppendorf tube:

- 1μL RBS + ALDH1 ZE
- 3μL dH2O
- 4μL enzyme master mix part A

Digest RBS + ALDH1 DL1 (57.47ng/μL) by adding the following to a labelled eppendorf tube:

- 1.74μL RBS + ALDH1 DL1
- 2.26μL dH2O
- 4μL enzyme master mix part A

Digest RBS + ALDH1 DE2 (conc. 74.62ng/μL) by adding the following to a labelled eppendorf tube:

- 1.34μL RBS + ALDH1 DE2
- 2.66μL dH2O
- 4μL enzyme master mix for part A

Digest RBS + ALDH1 AE (conc. 60.98ng/μL) by adding the following to a labelled eppendorf tube:

- 1.64μL RBS + ALDH1 AE
- 2.35μL dH2O
- 4μL enzyme master mix for part A

Digest RBS + ALDH1 AL1 (conc. 55.52 ng/μL) by adding the following to a labelled eppendorf tube:

- 1.8μL RBS + ALDH1 AL1
- 2.2μL dH2O
- 4μL enzyme master mix for part A

Digest RBS + ALDH1 AL2 (conc. 30.95ng/μL) by adding the following to a labelled eppendorf tube:

- 3.23μL RBS + ALDH1 AL2
- 0.77μL dH2O
- 4μL enzyme master mix for part A

Digest RBS + ALDH1 DL2 (conc. 30.95ng/μL) by adding the following to a labelled eppendorf tube:

- 3.23μL RBS + ALDH1 DL2
- 0.77μL dH2O
- 4μL enzyme master mix for part A

Digest RBS + ALDH1 DE1 (conc. 35.71ng/μL) by adding the following to a labelled eppendorf tube:

- 2.80μL RBS + ALDH1 DE1

- 1.20μL dH2O
- 4μL enzyme master mix for part A

Digest RBS + ALDH1 ZL2 (conc. 28.17ng/μL) by adding the following to a labelled eppendorf tube:
- 4μL RBS + ALDH1 ZL2
- 4μL enzyme master mix for part A

Digest D2 promoter (conc. 71.8 ng/μL) by adding the following to a labelled eppendorf tube:
- 1.39μL D2
- 2.61μL dH2O
- 4μL enzyme master mix part A

Digest PTAC promoter (conc. 53.2 ng/μL) by adding the following to a labelled eppendorf tube:
- 1.88μL PTAC
- 2.12μL dH2O
- 4μL enzyme master mix part A

Digest Terminator (conc. 90.8ng/μL) by adding the following to a labelled eppendorf tube:
- 1.10μL Terminator
- 2.89μL dH2O
- 4μL enzyme master mix for part B

Digest RBS + DBR2 A1 (conc. 67.7ng/μL) by adding the following to a labelled eppendorf tube:
- 1.48μL RBS + DBR2 A1
- 2.52μL dH2O
- 4μL enzyme master mix for part B

Incubate the digestions at 37^{o}C for 30 minutes in the water bath. Heat-kill at 80^{o}C for 20 minutes in the heat block.

Ligation:

Ligate the digested RBS + ALDH1 samples to the digested terminator by adding the following into a separate tube for each RBS + ALDH1 sample:
- 2μL of digested RFP Amp
- 2μL of the appropriate digested RBS + ALDH1 sample
- 2μL of the digested terminator
- 1μL of T4 DNA Ligase Reaction Buffer
- 0.5μL of T4 DNA ligase
- dH2O to 10μL

Ligate the digested promoters to the digested RBS + DBR2 by adding the following into a separate tube for each of the two promoters:
- 2μL of digested RFP Amp
- 2μL of the appropriate digested promoter
- 2μl of digested RBS + DBR2
- 1μL of T4 DNA Ligase Reaction Buffer
- 0.5μL of T4 DNA ligase

- dH2O to 10µL

Incubate the ligations at 16°C for 30 minutes and then 80°C for 20 minutes.

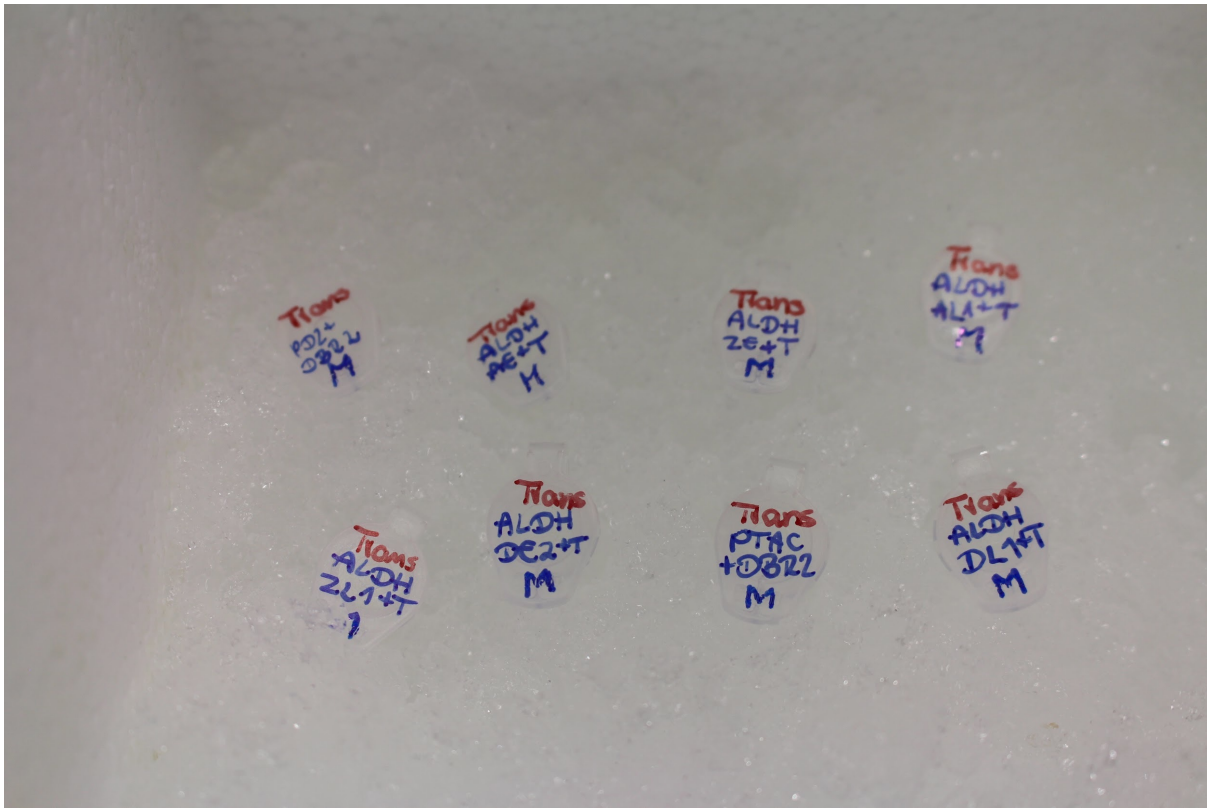
Transformation: (based on NEB High Efficiency Transformation protocol)

Objective: To transform the ligation product into DH5alpha cells.

Requirements:
- 50µL of competent cells per transformation
- Ligation product
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42°C
- Ice box
- Incubator
- 950µL of SOB medium per transformation
- Eppendorf tubes
- Bunsen burner
- Glass spreader
- Ethanol

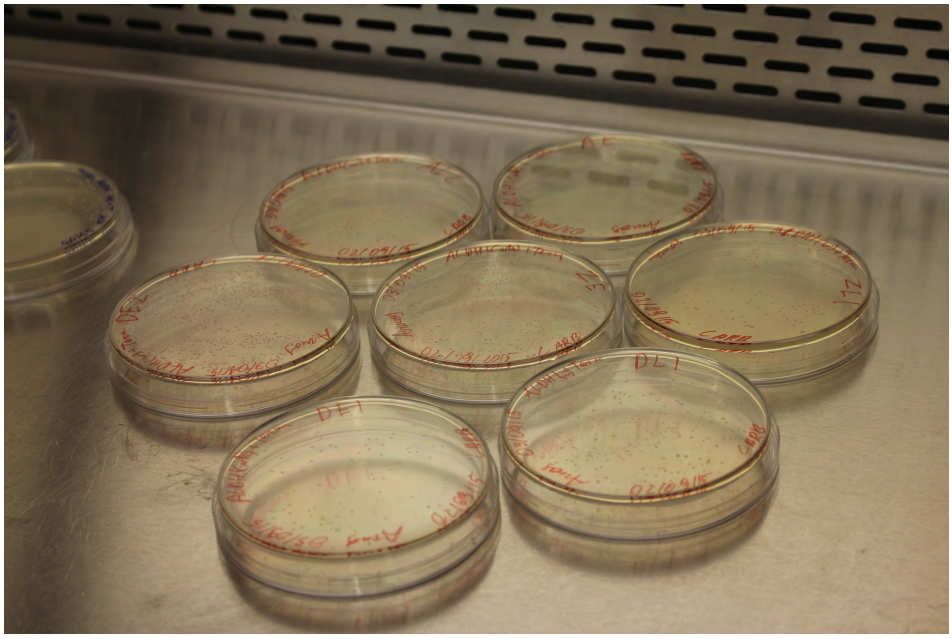
- Pre-chill eppendorf tubes on ice.
- Thaw competent cells on ice.
- Add 50µL of competent cells to each of the pre-chilled transformation tubes.
- Add 2µL of ligation product to its appropriate transformation tube.

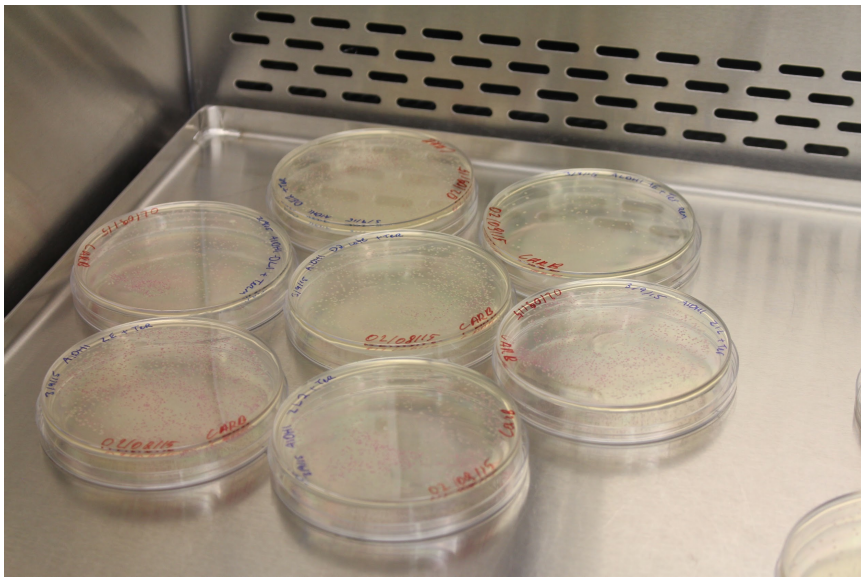
- Carefully flick the transformation tubes 4-5 times to mix the cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes.
- Heat-shock at 42°C in the heat-block for exactly 30 seconds.

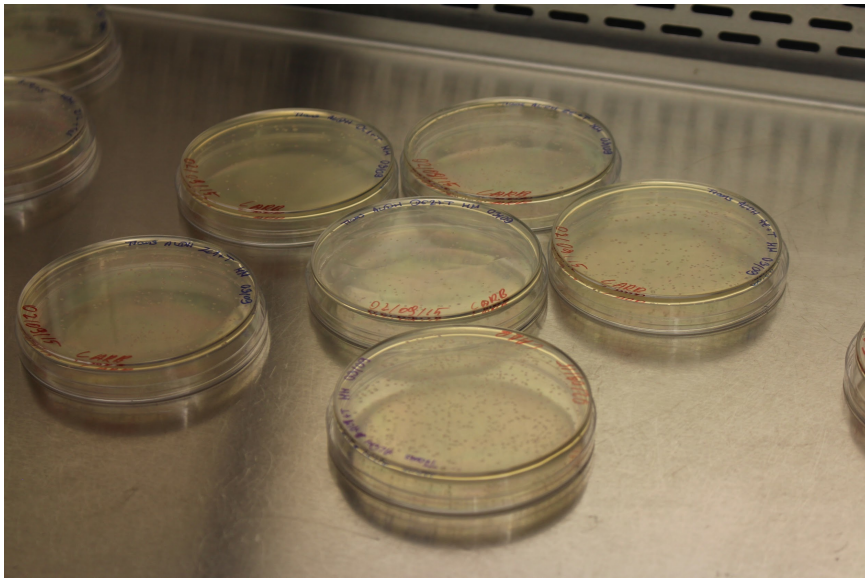


- Place the transformations on ice for 5 minutes.
- Pipette 950µL of SOB medium into each transformation tube.
- Incubate the transformations at 37°C for an hour with shaking.
- Warm selection plates to 37°C
- Spin the cells at 5g for 2 minutes.
- Remove 800µL of the supernatant from each tube and discard.
- Aspirate a number of times to resuspend the pellet before plating what's left in the transformation tube.
- Pass the glass spreader through the bunsen burner's flame before spreading.
- Cool the glass spreader on part of the plate that does not contain cells before spreading the cells on the plate.
- Incubate plates overnight at 37°C.

Results after 18 hours:







Broths 4th September:

Objective: to prepare broths from all of the transformation plates made the previous day.

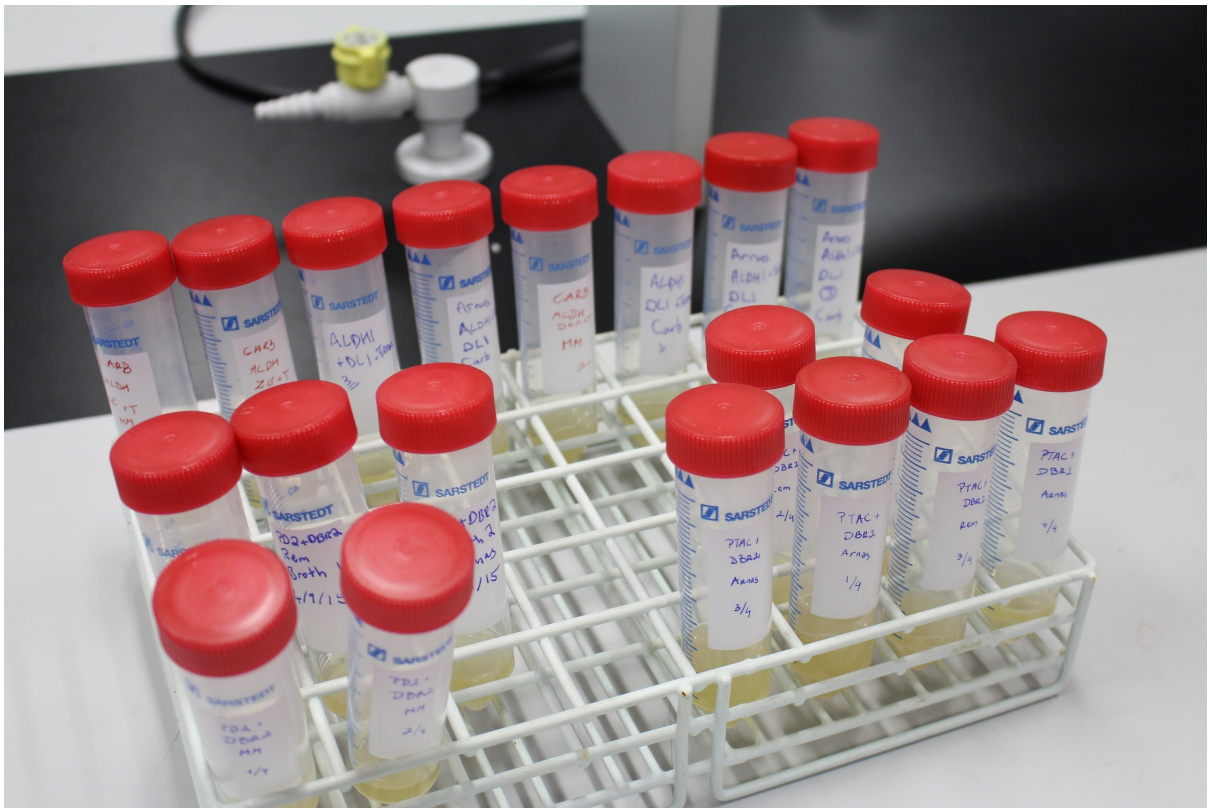
Requirements:

- 5mL LB medium per broth
- Appropriate antibiotic
- Tubes
- Incubator with shaker
- Transformation plates
- Wire loop
- Bunsen burner
- Permanent marker

Procedure: (perform beside a lighted bunsen burner)

- Add 5mL of LB medium to a falcon tube.
- Add 5µL of the appropriate 1000x antibiotic (carbenicillin).
- Use a sterile loop to scoop up a colony from the plate.
- Inoculate the LB medium in the falcon tube by placing the loop into the tube and stirring.
- Sterilise the loop by putting it into the bunsen burner's flame before repeating the process for the desired amount of broths.
- Incubate broths overnight at 37^{o}C with shaking.

Results:



The following broths were cloudy and not red:

PD2 + DBR2 MM Broth 1; 2
PD2 + DBR2 Arnas Broth 2
PD2 + DBR2 Remsha Broth 1; 4
PTAC + DBR2 Arnas Broth 1; 3; 4
PTAC + DBR2 Remsha Broth 2; 3; 4
ALDH1 AE + Terminator MM Broth 2
ALDH1 ZE + Terminator MM Broth 1
ALDH1 DL1 + Terminator Arnas Broth 1; 2; 3
ALDH1 DL1 + Terminator 1; 3
ALDH1 DL1 + Terminator MM 1

Miniprep 5th September:

(Protocol was identical to that followed on the 3rd of September except that 2mL of broth was miniprepped as opposed to 1mL).

Preparation of a 1% Agarose Gel 5th September:

Objective: To prepare a 1% Agarose Gel to run large PCR products ~ 1000bp to confirm part size.

Requirements:

- 0.75g Agarose
- ~400mL 1x TBE buffer
- 7.5µL GelRed
- Glass Bottle
- Gel Tray
- Electrodes
- Gel Comb
- P20 Pipette
- Pipette tips
- 25mL Pasteur Pipette
- Microwave

Procedure:

- Add 0.75g of agarose to a glass bottle.
- Add 75mL of 1x TBE Buffer.
- Swirl the solution and microwave until the agarose fully dissolves. Make sure to loosen the top of the glass bottle before placing it in the microwave. Be careful when handling the bottle. The solution should resemble water when the agarose is fully dissolved.
- When the solution is cool enough to touch, add 7.5µL of GelRed Nucleic Acid Gel Stain. Make sure to protect the GelRed from light when putting it back into storage.
- Swirl the glass bottle.
- Pour the gel into the gel tray.
- Remove bubbles using a pipette tip.
- Add the combs and leave the gel to solidify for a few hours.

Preparation of a 2% Agarose Gel 5th September:

Objective: To prepare a 2% Agarose Gel to run small PCR products ~ 200bp to confirm part size.

(protocol was identical to that carried out on 3rd September)

Polymerase Chain Reaction (PCR) 5th September:

Objective: To determine if purified plasmid DNA contains the desired part. (.ie if RBS + ALDH1 is ligated to a terminator; if Promoter D2 (BBa_K823008) is ligated to RBS + DBR2; if PTAC promoter (BBa_K864400) is ligated to RBS + DBR2.

Requirements:

- Q5® High-Fidelity 2X Master Mix
- Forward and Reverse Primers (primers came dry from Sigma)
- TE Buffer / Nuclease free H2O
- Ice box
- 96 well-plate
- Thermocycler
- Pipettes, pipette tips

Procedure (Based on NEB Protocol for Q5® High-Fidelity 2X Master Mix)

- Design primers in such a way as to determine by PCR product size if the previous ligation was carried out correctly.
- Get primers synthesised.
- Make a PCR master mix for each primer pair:

- **A W_ALDH1 Term LP and A W_ALDH1 Term RP:** (To be run on 2% agarose gel)

(Primers designed so that if terminator has successfully been ligated to RBS+ALDH1 the PCR product will be 208bp long; if terminator isn't present PCR product will be 61bp long) There are eight miniprepped samples. Create master mix for ten samples to allow for pipetting error. Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:

- ❏ 125µL Q5® High-Fidelity 2X Master Mix
- ❏ 12.5µL of 10µM (add 10µL of 100µM forward primer to 90µL of TE Buffer in a separate tube) forward primer
- ❏ 12.5µL of 10µM reverse primer
- ❏ 80µL TE Buffer

- **PTAC+DBR2 LP and PTAC+DBR2 RP:** (To be run on 2% agarose gel)

(Primers designed so that if PTAC has been successfully ligated to the RBS+DBR2 the PCR product will be 197bp long; if PTAC isn't present PCR product will be 117bp long) There are five miniprepped samples. Create master mix for seven samples to allow for pipetting error.

Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:

- ❏ 87.5µL Q5® High-Fidelity 2X Master Mix
- ❏ 8.75µL of 10µM (add 1µL of 100µM forward primer to 9µL of TE Buffer in a separate tube) forward primer
- ❏ 8.75µL of 10µM reverse primer
- ❏ 56µL TE Buffer

- **A_p23106_S+DBR2 LP and A_p23106_S+DBR2 RP:** (To be run on 2% agarose gel)

(Primers designed so that if Promoter D2 (BBa_K823008) has been successfully ligated to RBS+DBR2 the PCR product size will be 134bp long; if PD2 isn't present the PCR product will be 82bp long) There are three minprepped samples. Create master mix for five reactions to allow for pipetting error. Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:

- ❏ 62.5µL Q5® High-Fidelity 2X Master Mix
- ❏ 6.25µL of 10µM (add 1µL of 100µM forward primer to 9µL of TE Buffer in a separate tube) forward primer
- ❏ 6.25µL of 10µM reverse primer
- ❏ 40µL TE Buffer

- **VF2 and VR:** (To be run on a 1% agarose gel)

(Based on PCR product size it is hoped that it can be determined whether or not the terminator had ligated successfully in the case of RBS+ALDH1 and if the promoters had ligated successfully to RBS+DBR2) There is a total of 16 miniprepped samples. Create master mix for 18 reactions to allow for pipetting error. Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:

- ❏ 225µL Q5® High-Fidelity 2X Master Mix
- ❏ 22.5µL of 10µM (add 10µL of 100µM forward primer to 90µL of TE Buffer in a separate tube) forward primer
- ❏ 22.5µL of 10µM reverse primer
- ❏ 144µL TE Buffer

- ■ Add 23µL of the appropriate master mix to pre-planned wells of the 96 well-plate.
- ■ Add 2µL of template DNA to its corresponding well.
- ■ Seal the well-plate.
- ■ Transfer the well plate to the thermocycler.
- ■ Use the NEB Tm Calculator to determine the annealing temperature.
- ■ Set the program on the thermocycler:
- ❏ Initial Denaturation: 98^{o}C for 30 seconds
- ❏ 32 cycles: 98^{o}C for 5-10 seconds; 62^{o}C for 10-30 seconds; 72^{p}C for 20-30 seconds.
- ❏ Final Extension: 72^{o}C for 2 minutes.

Running PCR products on a 1% and 2% agarose gels 5th September:

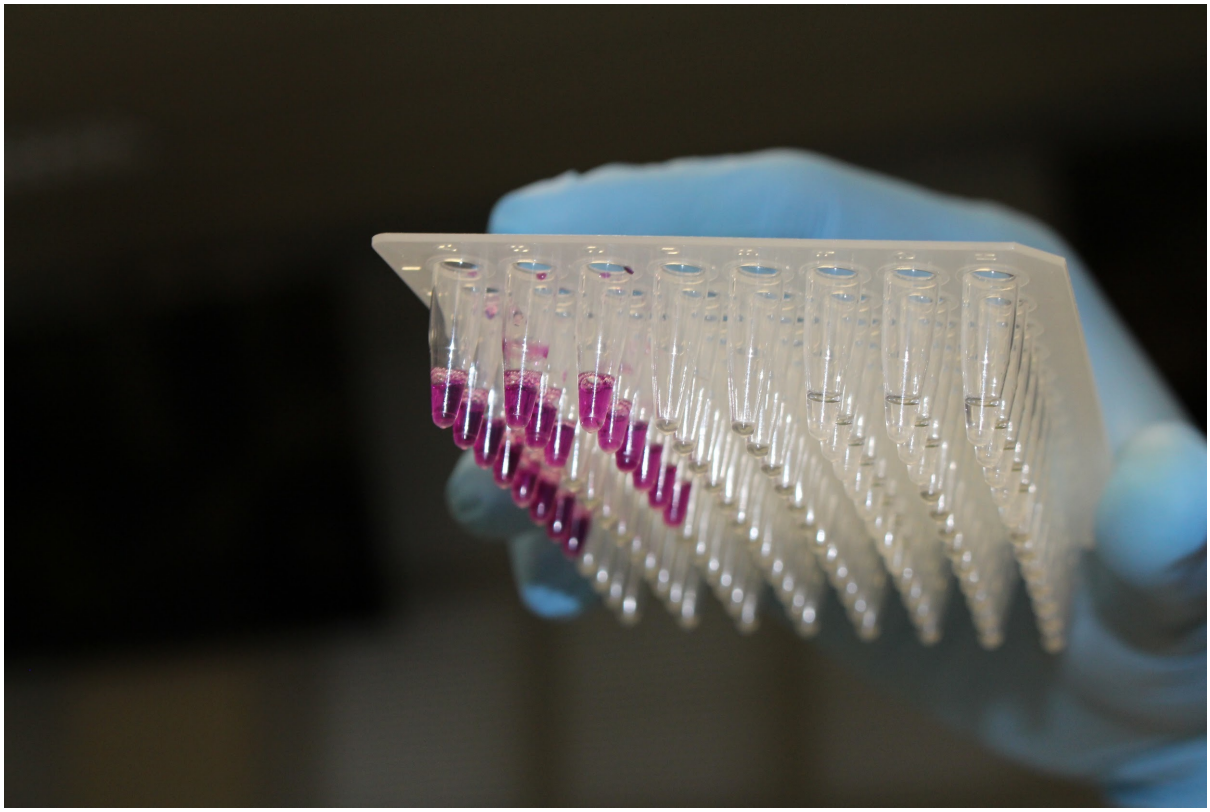
Objective: To investigate if PCR products are the desired size, to determine whether or not previous ligation had been successful.

Requirements:

- 1% and 2% agarose gels
- 1x TBE buffer
- PCR products
- NEB Gel Loading Dye, Purple 6x
- NEB Quick-Load® Purple 2-Log DNA Ladder (0.1 - 10.0 kb)
- P20 calibrated pipette, pipette tips
- Electrodes
- Gel cover
- Power source
- UV light source

Procedure:

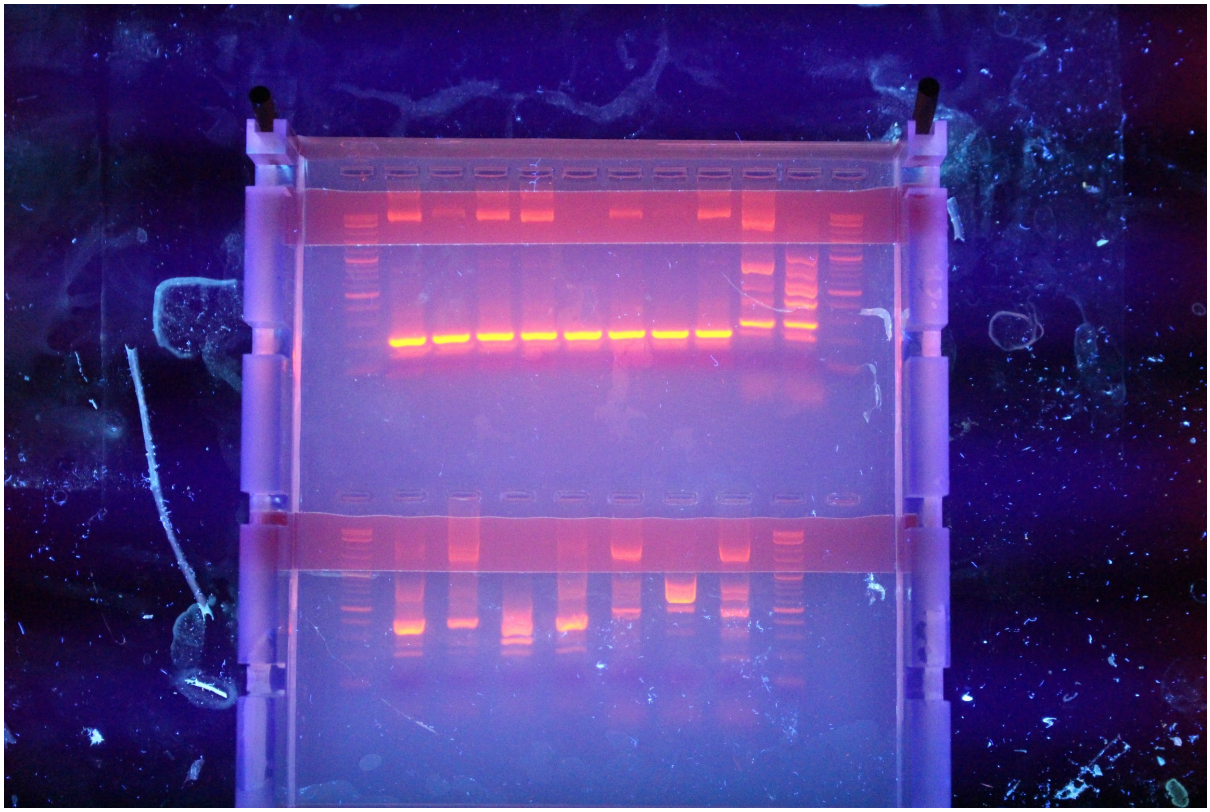
- Prepare a 1% and 2% agarose gel.
- When gels have set, position them correctly in the gel trays and remove the combs.
- Submerge gel in 1x TBE buffer.
- Add 5µL of NEB Gel Loading Dye, Purple 6x to each PCR well containing a sample.



- Prepare a number of Quick-Load® Purple 2-Log DNA Ladder (0.1 - 10.0 kb) samples to act as a reference for size.
- Create a plan as to which lane each sample will run.
- Add 15µL of each sample to its appropriate lane in the gels.
- Place the covers on the gels.
- Attach the electrodes, remembering that the DNA will run towards the red (positive) electrode.

- Run the 2% gel at a low voltage (60-80V) for a few minutes. Run the 1% gel at ~100 volts for a few minutes.
- Look at the gels under UV light. Compare sample sizes to DNA Ladder to determine if ligation was carried out successfully.

Results:



2% agarose gel: ran PCR products deriving from the primers A W_ALDH1 Term LP and A W_ALDH1 Term RP; PTAC+DBR2 LP and PTAC+DBR2 RP; A_p23106_S+DBR2 LP and A_p23106_S+DBR2 RP.

Top row:
Lane 1: DNA Ladder
Lane 2: A ALDH1 DL1 + Terminator
Lane 3: A ALDH1 DL1 + Terminator 1//
Lane 4: ALDH1 AE + Terminator MM
Lane 5: ALDH1 + Terminator DL1 Arnas
Lane 6: ALDH1 DL1 + Terminator 3
Lane 7: ALDH1 DL1 + Terminator Arnas 3
Lane 8: ALDH1 DL1 + Terminator MM 1

Lane 9: PTAC + DBR2 Arnas 1
Lane 10: PTAC + DBR2 Arnas 3
Lane 12: DNA Ladder

Bottom Row:
Lane 1: DNA Ladder
Lane 2: PTAC + DBR2 Remsha 2
Lane 3: PTAC + DBR2 Remsha 3
Lane 4: PTAC + DBR2 Arnas 4
Lane 5: PTAC + DBR2 Arnas 3 Repeat
Lane 6: PD2 + DBR2 Remsha 1
Lane 7: PD2 + DBR2 Remsha 4
Lane 8: PD2 + DBR2 Arnas 2
Lane 9: DNA Ladder

1% agarose gel: ran PCR products from VF2 and VR primers

Top Row:
Lane 1: DNA Ladder
Lane 2: PTAC + DBR2 Arnas 1
Lane 3: PTAC + DBR2 Arnas 3
Lane 4: PTAC + DBR2 Remsha 2
Lane 5: PTAC + DBR2 Remsha 3
Lane 6: PTAC + DBR2 Arnas 4
Lane 7: PD2 + DBR2 Remsha 1
Lane 8: PD2 + DBR2 Remsha 4

Lane 9: PD2 + DBR2 Arnas 2 Lane 10: DNA Ladder

Bottom Row:
Lane 1: DNA Ladder Lane 2: A ALDH1 DL1 + Terminator
Lane 3: A ALDH1 DL1 + Terminator 1// Lane 4: ALDH1 AE + Terminator MM
Lane 5: ALDH1 + Terminator DL1 Arnas Lane 6: ALDH1 DL1 + Terminator 3
Lane 7: ALDH1 DL1 + Terminator Arnas 3 Lane 8: ALDH1 DL1 + Terminator MM 1
Lane 9: DNA Ladder

Conclusions:

From the 2% gel, all eight of the RBS+ALDH1+Terminator samples had bands at ~200bp. From the 1% gel, all eight samples have bands at ~2000bp. In the case of the 2% gel if the terminator had been properly ligated the expected length of the PCR product was 208bp (if the terminator hadn't ligated, the expected length was 61bp). In the 1% gel the expected length of the bands was ~2000bp. (http://parts.igem.org/Help:Primers/Problems_using_VF2_and_VR_to_amplify_BioBrick_parts offers an explanation as to the reason for the presence of a ~200bp band in all of the 1% gel RBS+ALDH1+Terminator samples. "VR can potentially bind to BBa_B0010. The popular terminator BBa_B0015 contains BBa_B0010. If VR anneals to B0010..., a 183 bp band would be produced by the PCR". The terminator we used to ligate to RBS+ALDH1 was BBa_B0015). From the results of both the 1% and 2% gel we determined that the ligation of the terminator had been successful. We thought that the sample in Lane 6 of the top row of the 2% gel (ALDH1 DL1 + Terminator 3) had the clearest band and would be the best to proceed with but due to its low ng/μL concentration from its miniprep we decided to use the sample in Lane 3 of the 2% gel (A ALDH1 DL1 + Terminator 1//) in future ligations.

From the 2% gel all of PTAC+RBS+DBR2 PCR products showed multiple bands with all of their brightest bands being longer than 200bp. If PTAC had been ligated successfully the expected product length would have been 197bp (the expected product length if the ligation had been unsuccessful was 117bp). Comparing the results on the 2% gel to the expected results it appeared that in all cases the ligation of PTAC to RBS+DBR2 had been unsuccessful. None of the PTAC+RBS+DBR2 samples on the 1% gel showed bands of the expected length (~1200bp) also.

From the 2% gel all of the Promoter D2+RBS+DBR2 samples showed multiple bands and their brightest bands were approximately at the 500bp mark. The expected product length of Promoter D2+RBS+DBR2 was 134bp (if the ligation had been unsuccessful the PCR product was expected to be 82bp). Again comparing the results on the gel to the expected results it appeared that the ligation had been unsuccessful. The sample in the top row Lane 8 of the 1% gel (PD2 + DBR2 Remsha 4) showed a band at the expected length (~1200bp) but also showed another strong band.

Planning based on results:

It had been planned to ligate promoter+RBS+DBR2 to RBS+ALDH1+Terminator but based on the results of the gels it was decided not to proceed as based on the evidence it appeared that none of the promoters had successfully ligated to RBS+DBR2. We discussed our options. Based on the last set of sequencing results it seemed that the step of ligating a promoter to RBS+DBR2 had also been a problem.

We decided that we would attempt to ligate all the promoters that we had prepared to RBS +DBR2 and in parallel to ligate all of the promoters to the RBS+ALDH1+Terminator 1// sample that we thought had been successfully assembled.

Digestion and Ligation 6th September:

Objective: To ligate the promoters; D1 (Device 1 promoter interlab study K823005), D2 (K823008), D3 (K823013), PTAC (K864400) and PLAC (K864400) to RBS+DBR2 and RBS +ALDH1+Terminator.

Requirements:

- NEBuffer 2.1
- Eco-RI HF®
- PstI
- SpeI
- XbaI
- dH2O
- Pipettes, pipette tips
- Water bath and/or heat block/thermocycler
- eppendorf tubes
- permanent marker
- T4 DNA ligase reaction buffer
- T4 DNA ligase
- ice box

Procedure (based on iGEM protocol http://parts.igem.org/Help:Protocols/3A_Assembly)

- Calculate the required amount of enzyme master mixes.

Prepare Enzyme master mix for plasmid backbone by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL PstI
- 18.5µL dH2O

Prepare Enzyme master mix for Part A by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL SpeI
- 18.5µL dH2O

Prepare Enzyme master mix for Part B by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL PstI
- 0.5µL XbaI

- 18.5μL dH2O

Digestion:

Digest RFP kanamycin (BBa_J04450) (conc. 167.5 ng/μL) by adding the following to a labelled eppendorf tube:
- 0.6μL RFP kanamycin
- 3.4μL dH2O
- 4μL enzyme master mix for plasmid backbone

Digest RFP Ampicillin (BBa_J04450) (conc. 159.7ng/μL) by adding the following to a labelled eppendorf tube:
- 0.63μL RFP ampicillin
- 3.37μL dH2O
- 4μL enzyme master mix for plasmid backbone

Digest linearized tetracycline backbone (pSB1T3) (conc. 25 ng/μL) by adding the following to a labelled eppendorf tube:
- 4μL pSB1T3
- 4μL enzyme master mix for plasmid backbone

Digest promoter D1 (in chloramphenicol backbone) (conc. 93 ng/μL) by adding the following to a labelled eppendorf tube:
- 1.08μL D1
- 2.92μL dH2O
- 4μL enzyme master mix for part A

Digest promoter D2 (in chloramphenicol backbone) (conc. 93.6 ng/μL) by adding the following to a labelled eppendorf tube:
- 1.07μL D2
- 2.93μL dH2O
- 4μL enzyme master mix for part A

Digest promoter D3 (in chloramphenicol backbone) (conc. 93.2ng/μL had been checked twice using nanodrop and had varied each time) by adding the following to a labelled eppendorf tube:
- 1μL D3
- 3μL dH2O
- 4μL enzyme master mix for part A

Digest PTAC (in chloramphenicol backbone) (conc. 53.2 ng/μL) by adding the following to a labelled eppendorf tube:
- 1.88μL PTAC
- 2.12μL dH2O
- 4μL enzyme master mix for part A

Digest PLAC (in ampicillin backbone) (conc. 85.4 ng/μL) by adding the following to a labelled eppendorf tube:
- 1.2μL PLAC
- 2.8μL dH2O

- 4μL enzyme master mix for part A

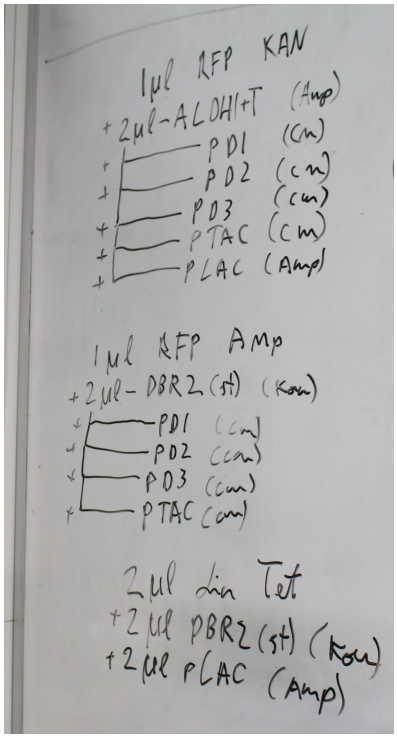
Digest RBS+ALDH1+Terminator 1// (1// refers to specific sample's name) (in ampicillin backbone) (conc. 82.3 ng/μL) by adding the following to a labelled eppendorf tube:

- 1.2μL RBS+ALDH1+Terminator 1//
- 2.8μL dH2O
- 4μL enzyme master mix for part B

Digest RBS+DBR2 A1 (A1 refers to specific sample's name, sequence had been confirmed) (in kanamycin backbone) (conc. 67.7 ng/μL) by adding the following to a labelled eppendorf tube:

- 1.48μL RBS+DBR2 A1
- 2.52μL dH2O
- 4μL enzyme master mix for part B

Incubate the digestions at 37°C for 30 minutes. Heat-kill the samples at 80°C for 20 minutes.



Ligation:

Ligate the digested promoters to the digested RBS+ALDH1+Terminator 1// by adding the following to new eppendorf tubes:

- 1μl digested RFP kanamycin
- 2μL of the appropriate digested promoter
- 2μL of digested RBS+ALDH1+Terminator 1//
- 1μL T4 DNA ligase reaction buffer
- 0.5μL T4 DNA ligase
- 3.5μL dH2O

Ligate the digested promoters to the digested RBS+DBR2 A1 by adding the following to new eppendorf tubes:

- 1µL digested RFP Ampicillin
- 2µL of the appropriate digested promoter
- 2µL of digested RBS+DBR2 A1
- 1µL T4 DNA ligase reaction buffer
- 0.5µL T4 DNA ligase
- 3.5µL dH2O

Ligate the digested PLAC promoter to the digested RBS+DBR2 A1 by adding the following to a new eppendorf tube:

- 2µL digested linearized tetracycline backbone pSB1T3
- 2µL digested PLAC
- 2µL digested RBS+DBR2 A1
- 1µL T4 DNA ligase reaction buffer
- 0.5µL T4 DNA ligase
- 2.5µL dH2O

Incubate the ligations at 16^{o}C for 30 minutes. Heat-kil at 80^{o}C for 20 minutes.

Transformation: (based on NEB High Efficiency Transformation protocol) 6th September

Objective: To transform the ligated products into DH5alpha cells so that purified DNA can be obtained via plating, broth and miniprep. Also to transform the hypothetical functioning device of promoter+RBS+ALDH1+Terminator directly into B569 cells. B569 cells were obtained from Amyris who engineered the cells to produce artemisinic acid, a precursor to artemisinin. The aim is then to put the transformed B569 cells with the device into a fermenter for a number of days and to carry out GCMS analysis to check levels of artemisinic acid versus a control of B569 cells without the device.

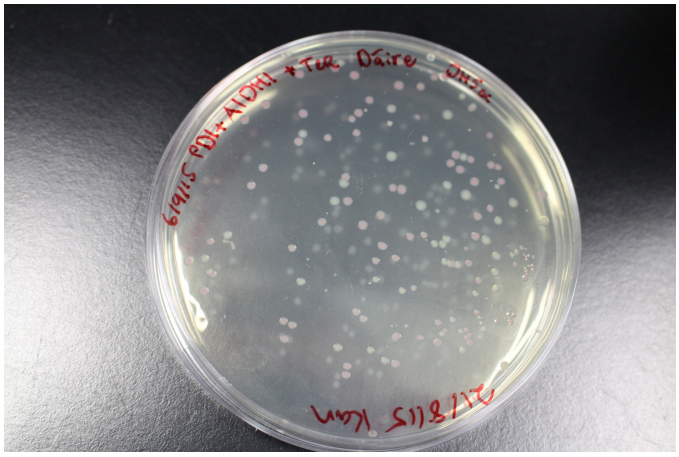
- Pre-chill eppendorf tubes on ice.
- Thaw competent cells on ice.
- Add 50µL of the appropriate strain of competent cells to each of the pre-chilled transformation tubes.
- Add 2µL of ligation product to its appropriate transformation tube.
- Carefully flick the transformation tubes 4-5 times to mix the cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes.
- Heat-shock at 42^{o}C in the heat-block for exactly 30 seconds.
- Place the transformations on ice for 5 minutes.
- Pipette 950µL of SOB medium into each transformation tube.
- Incubate the transformations at 37^{o}C for an hour with shaking.
- Warm selection plates to 37^{o}C. Use kanamycin plates for the ALDH1 device in DH5alpha cells. Use carbenicillin plates for D1, D2, D3 and PTAC ligation products with RBS+DBR2. Use tetracycline plate for PLAC+RBS+DBR2 ligation product. Use carbenicillin, chloramphenicol and kanamycin triple antibiotic plates for ALDH1

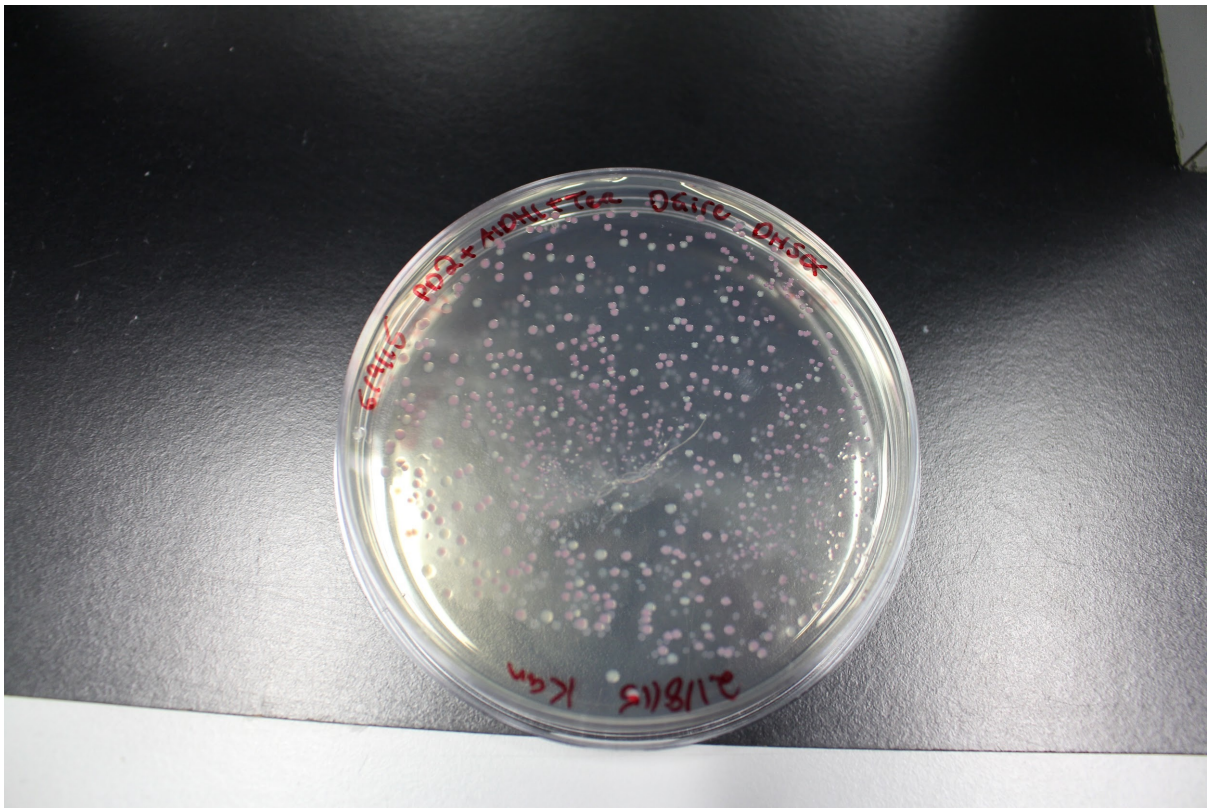
device transformation into B569 cells (B569 strain originally has two plasmids, giving resistance to carbenicillin and chloramphenicol).

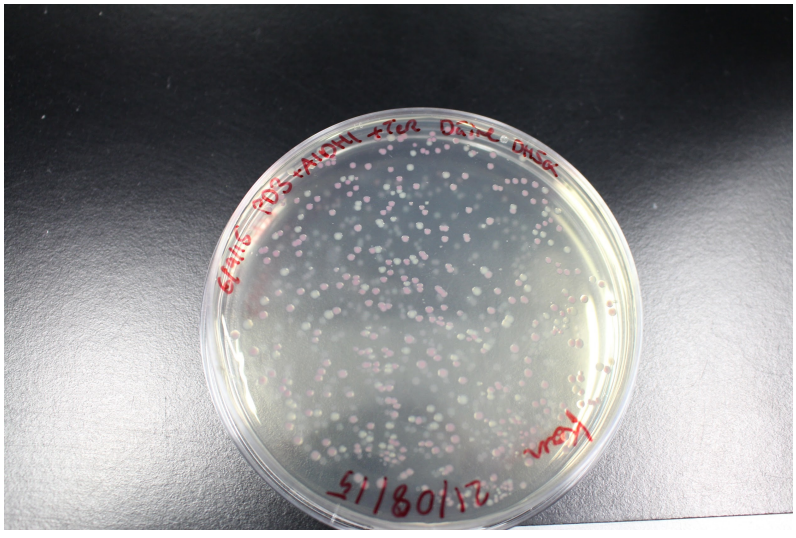
- Spin the cells at 5g for 2 minutes.
- Remove 800µL of the supernatant from each tube and discard.
- Aspirate a number of times to resuspend the pellet before plating what's left in the transformation tube.
- Pass the glass spreader through the bunsen burner's flame before spreading.
- Cool the glass spreader on part of the plate that does not contain cells before spreading the cells on the plate.
- Incubate plates overnight at 37^{o}C.

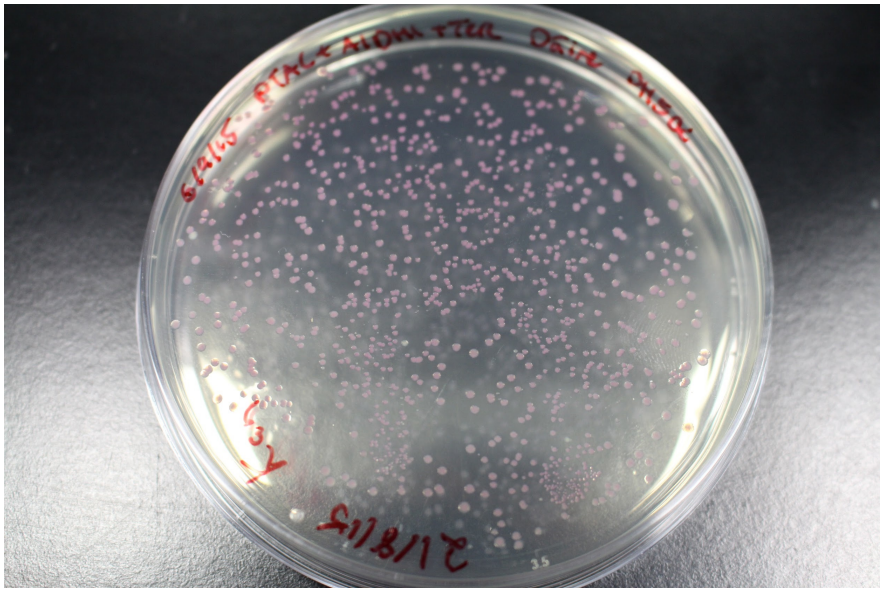
Results: Plates after ~18 hours at 37^{o}C:

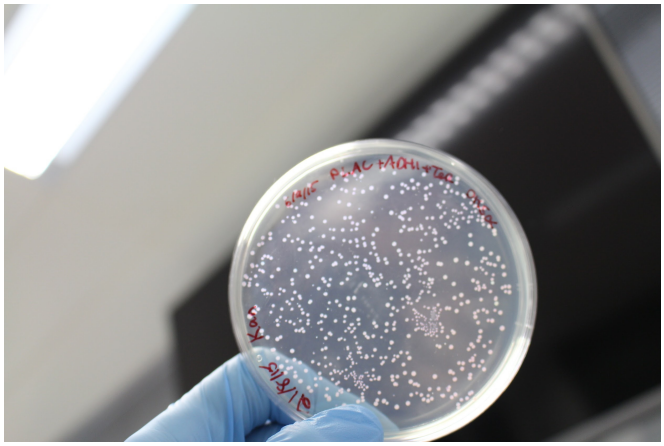
All B569 plates had less growth than their DH5alpha equivalents that had been transformed with the same device.

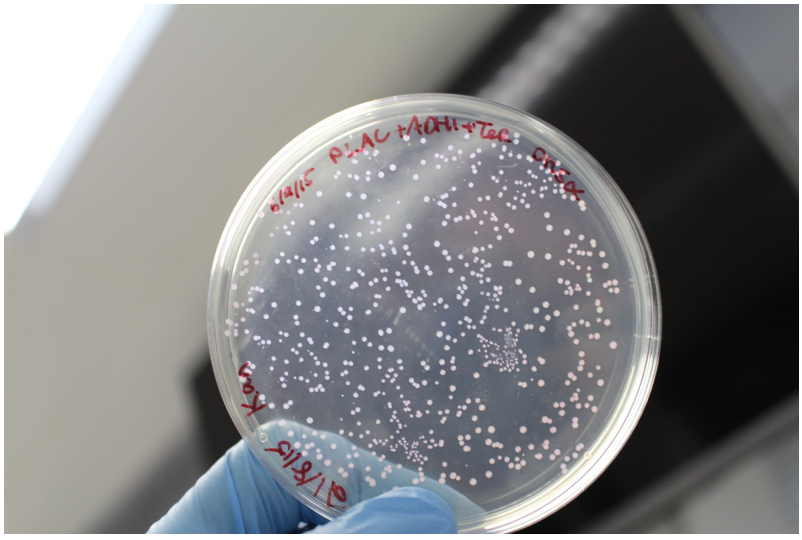


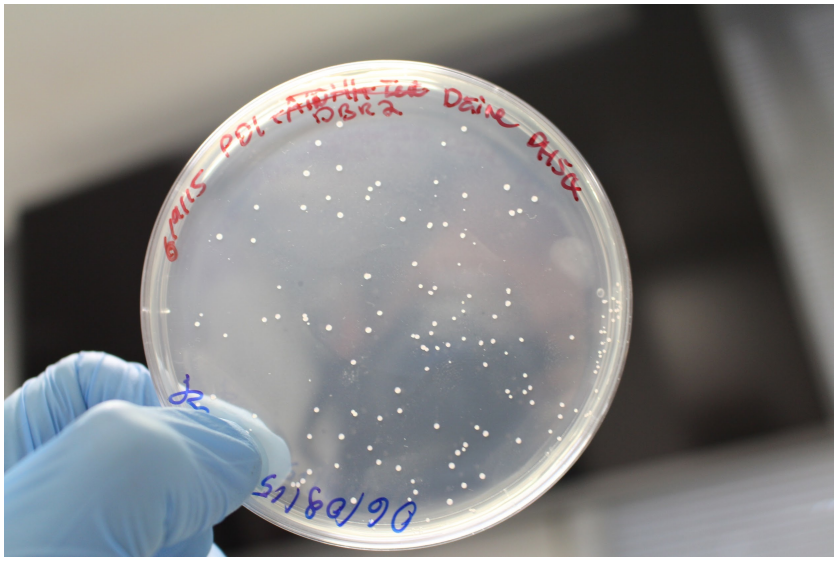


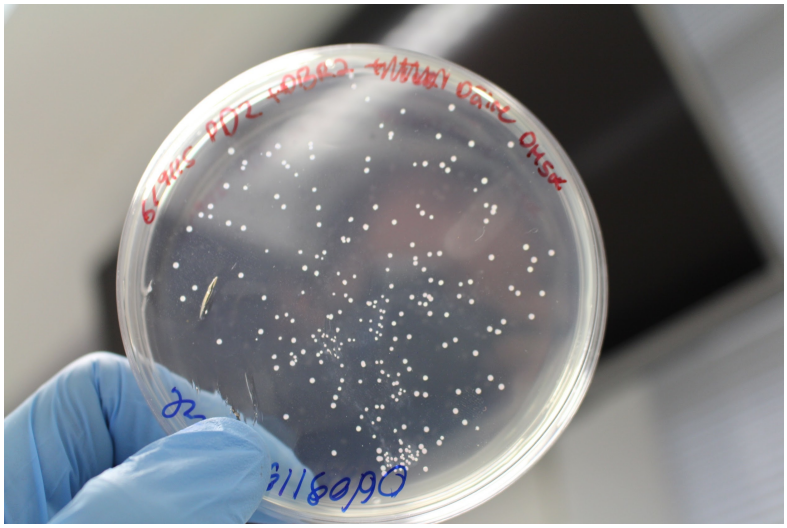


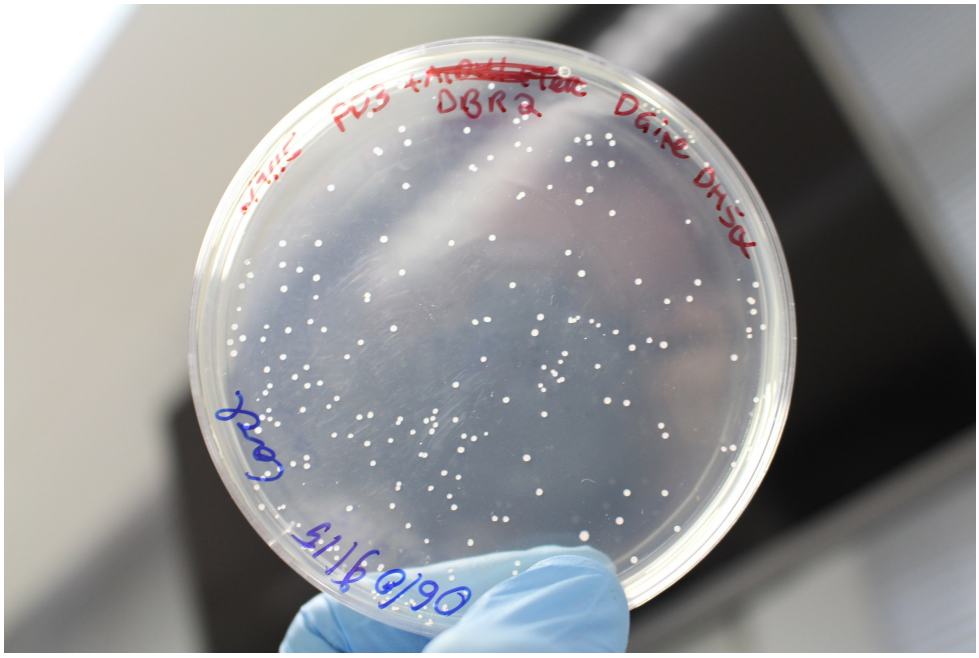


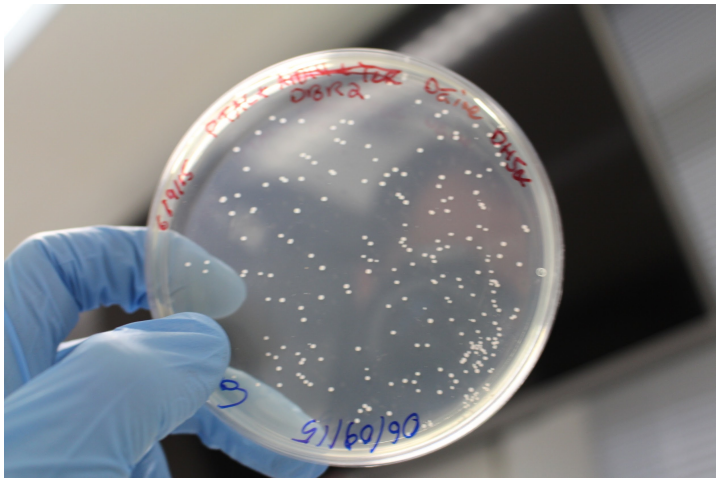


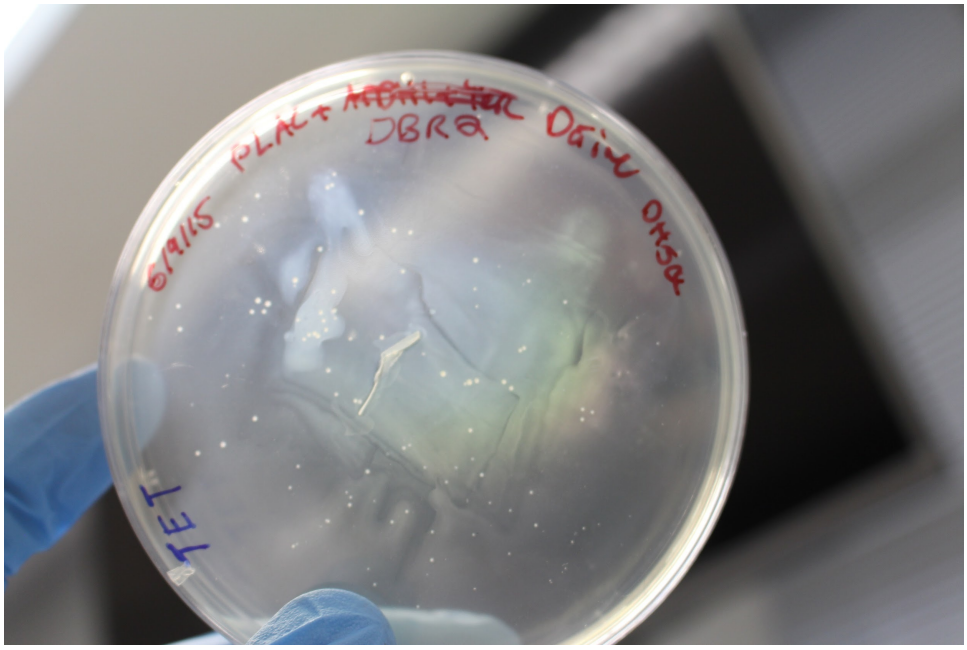


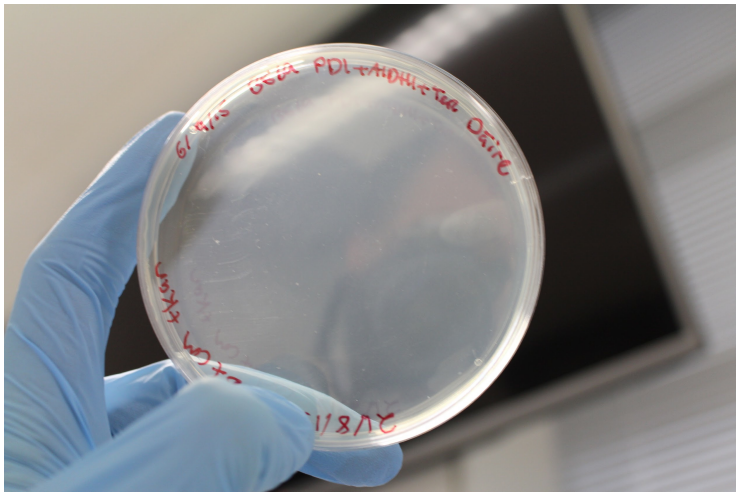


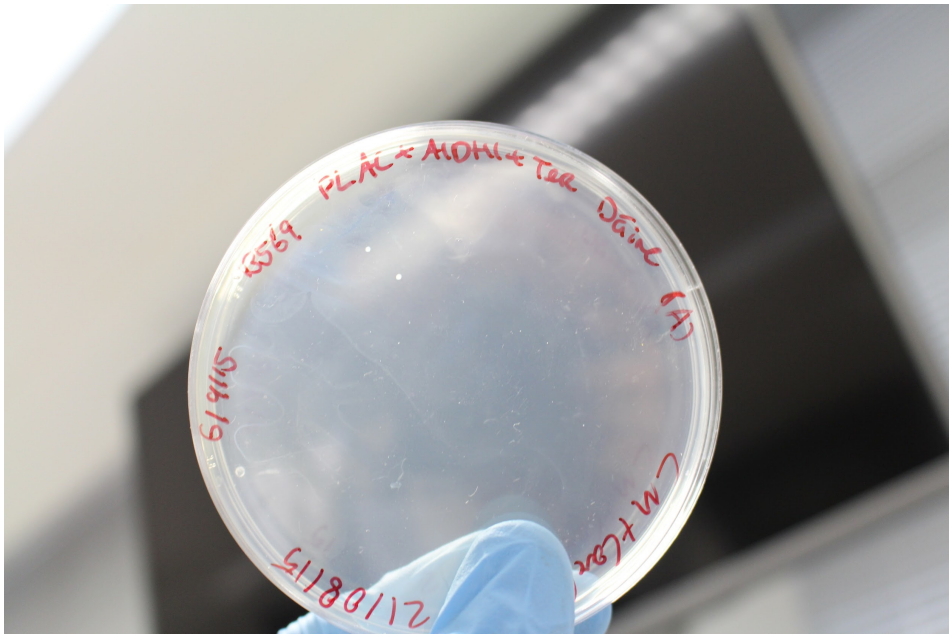


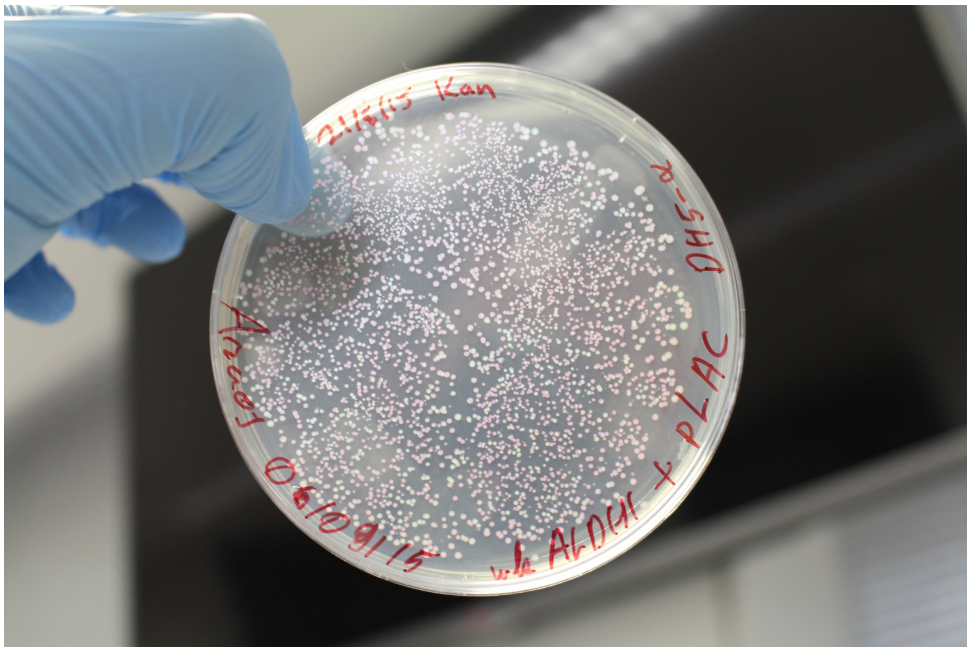


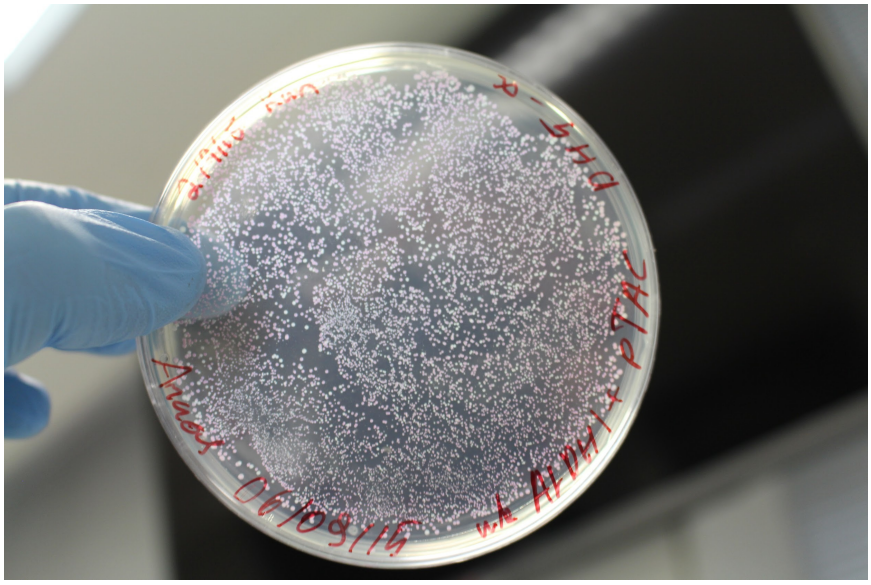

DBR2
6/9/15
9
5/16/09/15

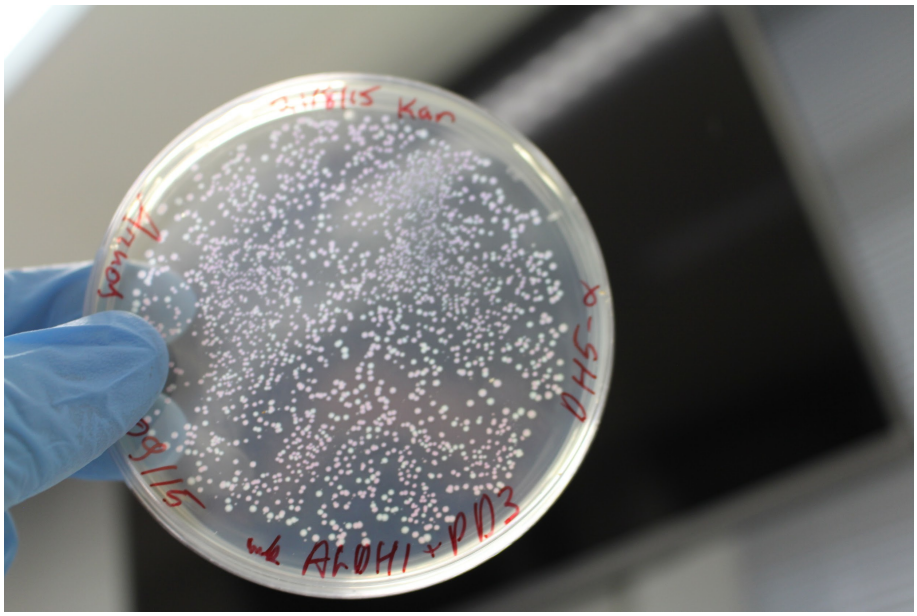

DBR2
6/9/15
TET

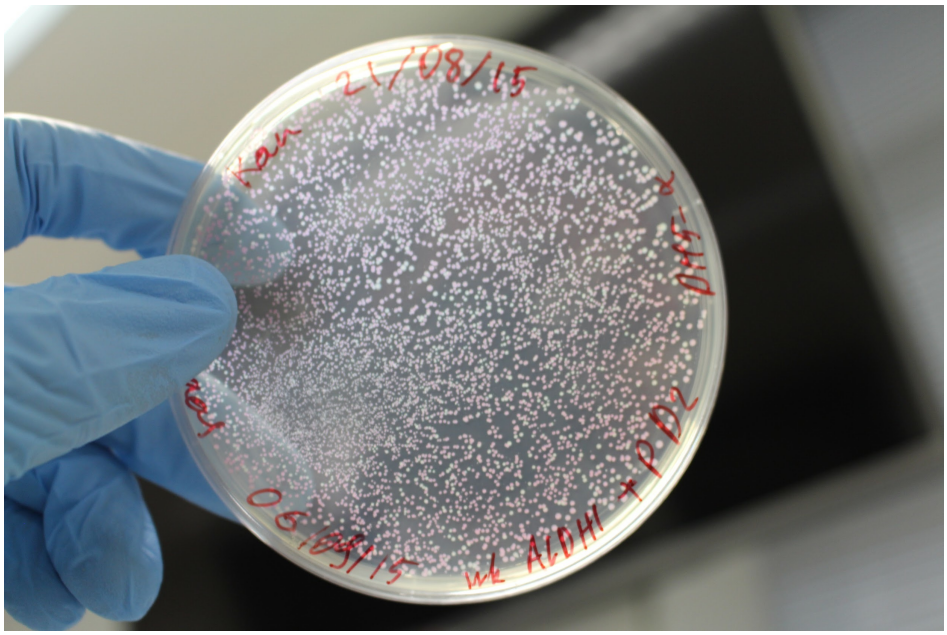


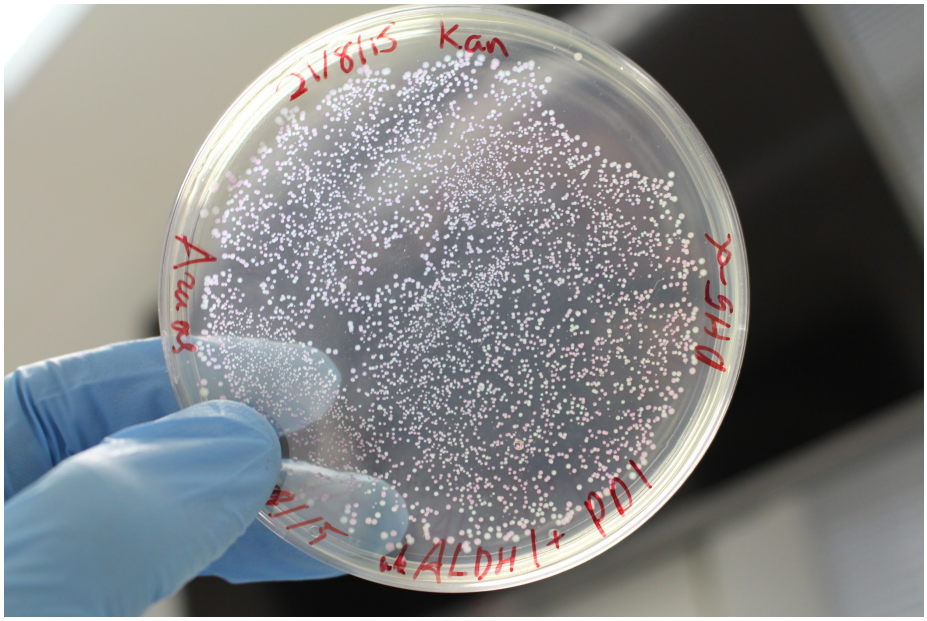

PLAC + ALDH + Tau
SHA19
5/18/15

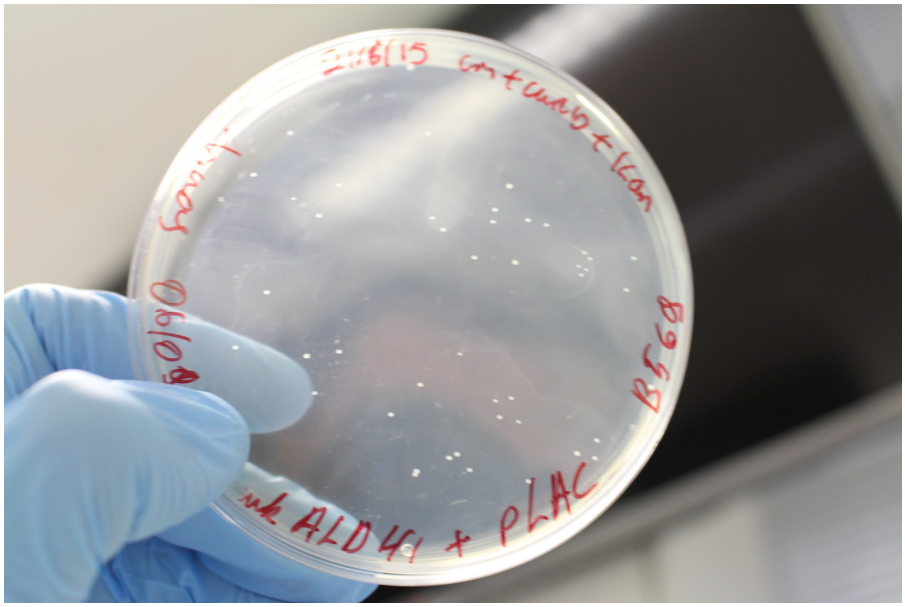

Kan
OHS-α
PLAC +
ALDH
6/09/15

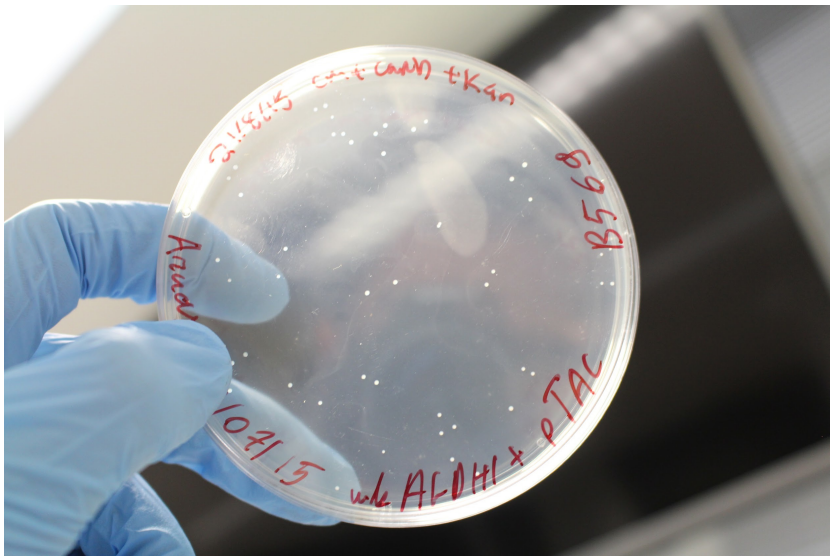

Amp
06/09/15
wt AlDH + pD2

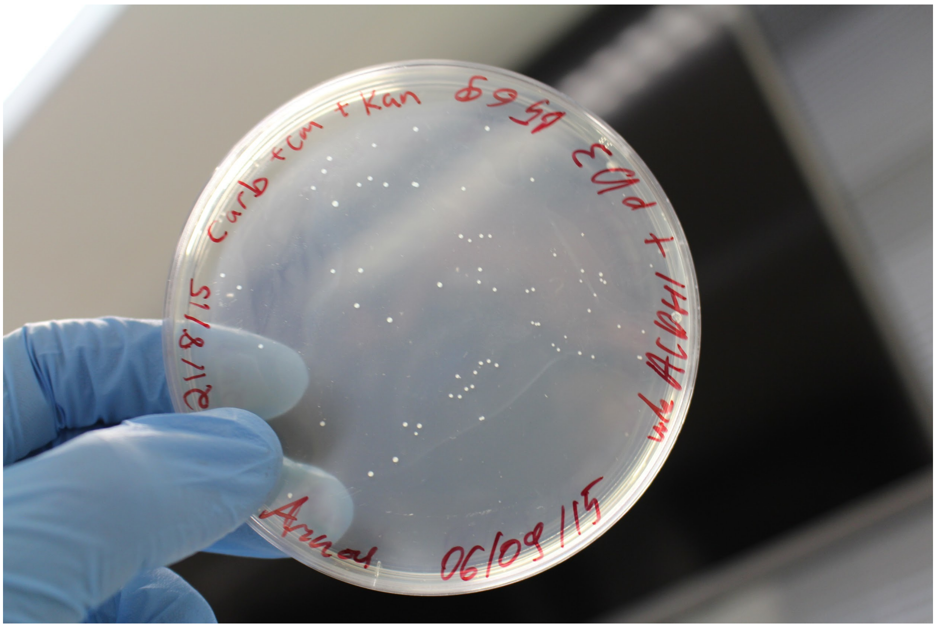

Kan
DH5-α
wt AlDH + pD3

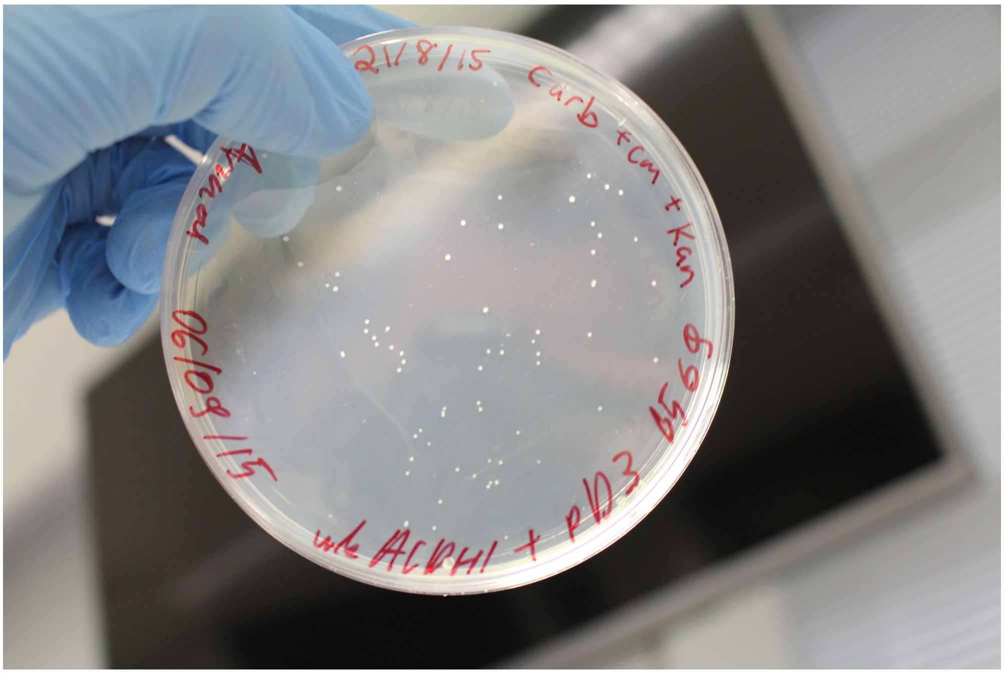

21/08/15
Kan
DH5-α
wt AlDH + pD2

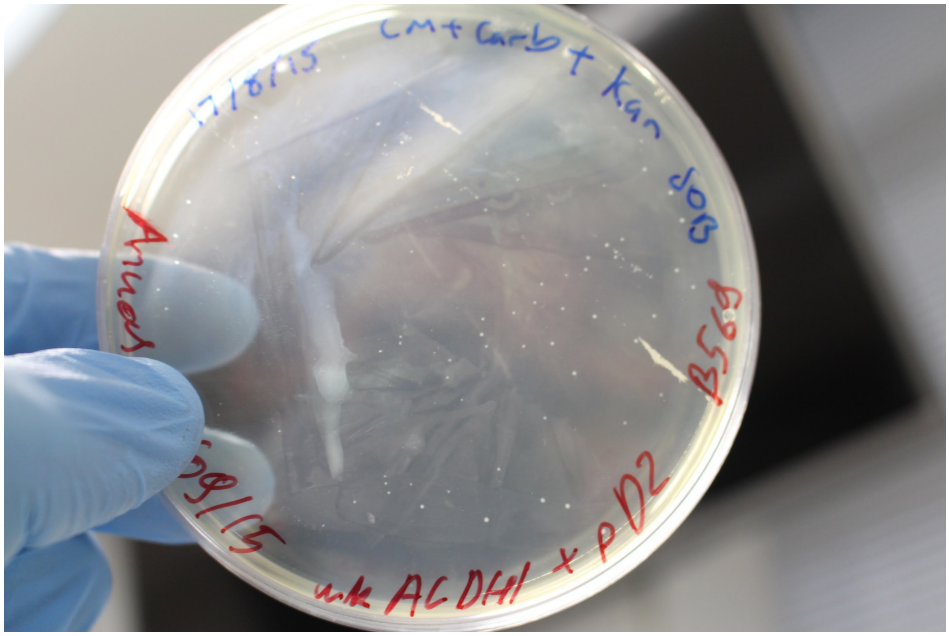

2/18/15 Kan
Arucas
pHSG
ALDH1 + PD1

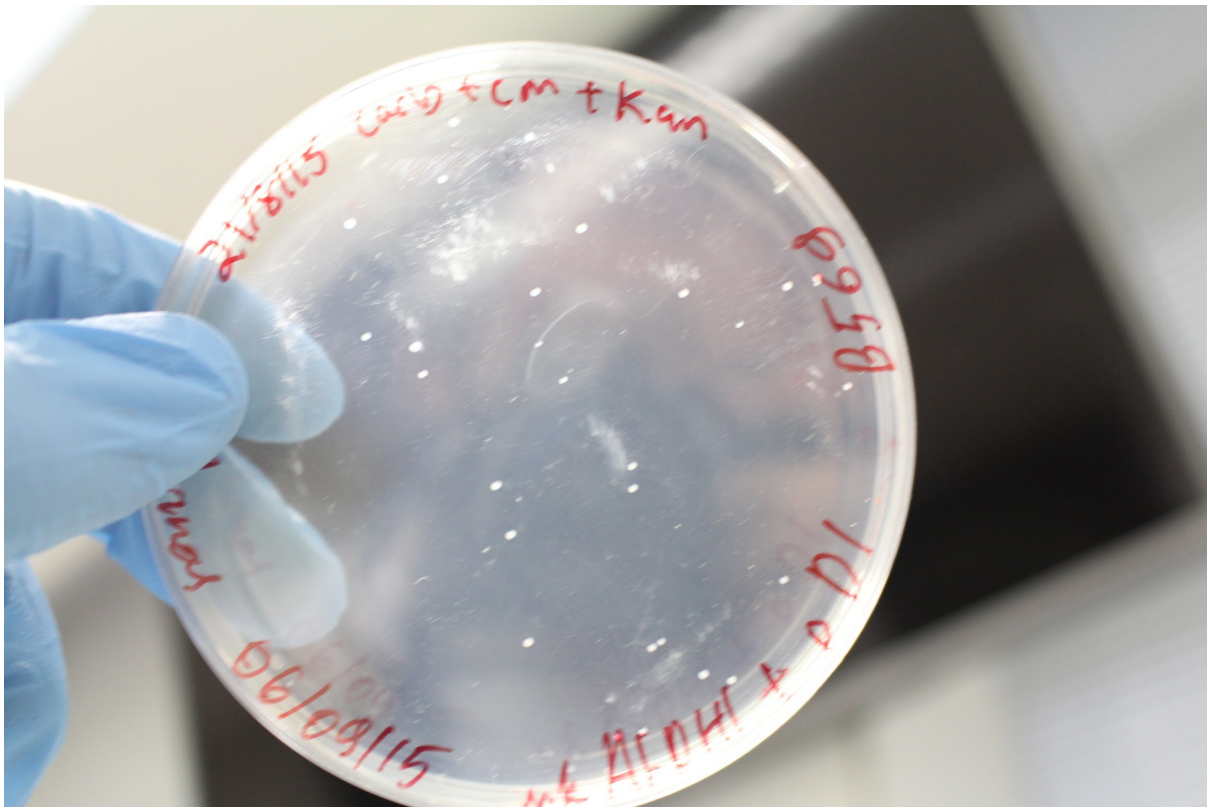

Cm + Carb + Kan
B598
ALD41 + pLAC

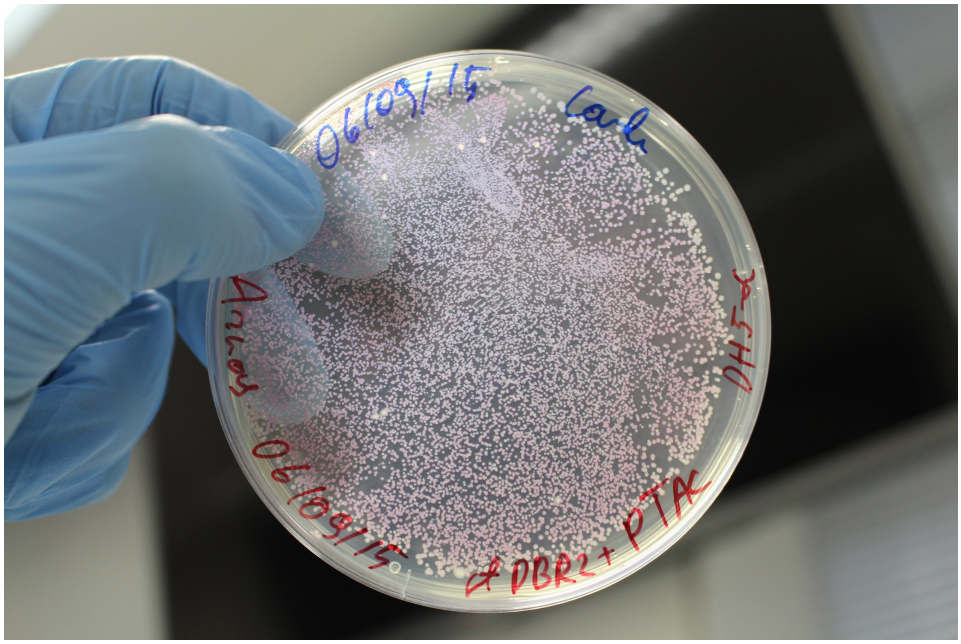

Carb + Kan
B576
10/1/15
ALDH1 + pTAC

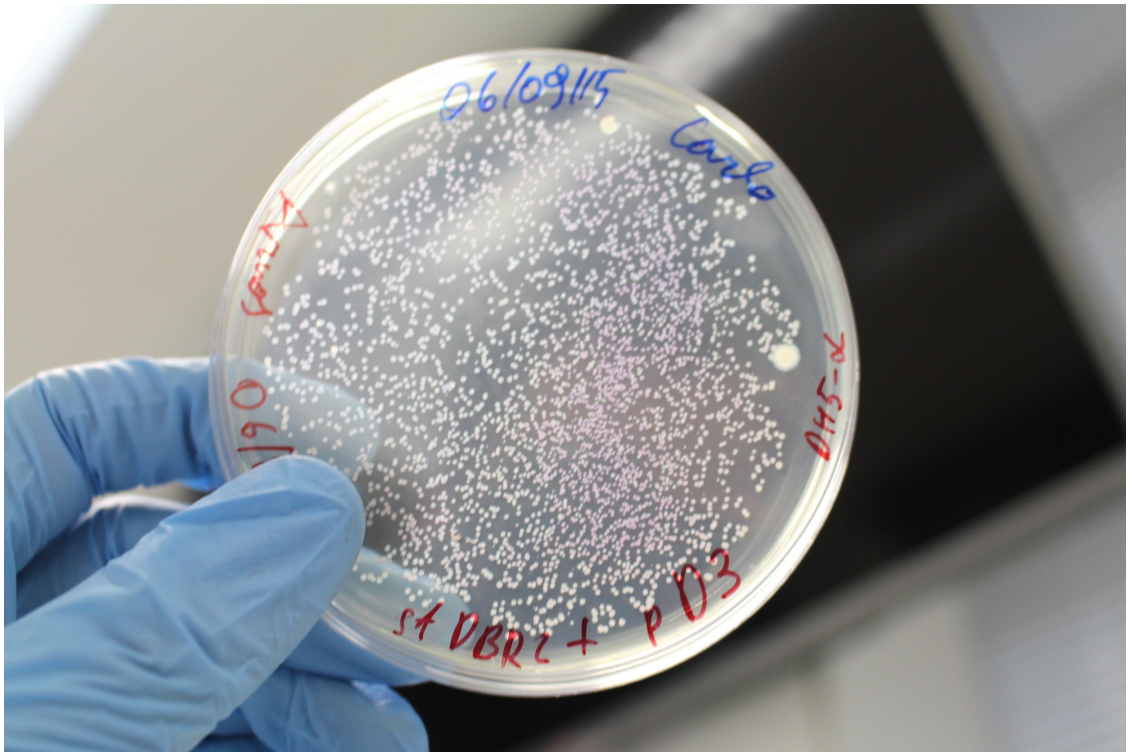

Carb +Cm +Kan
21/8/15
06/09/15
w/e ACRH1 +

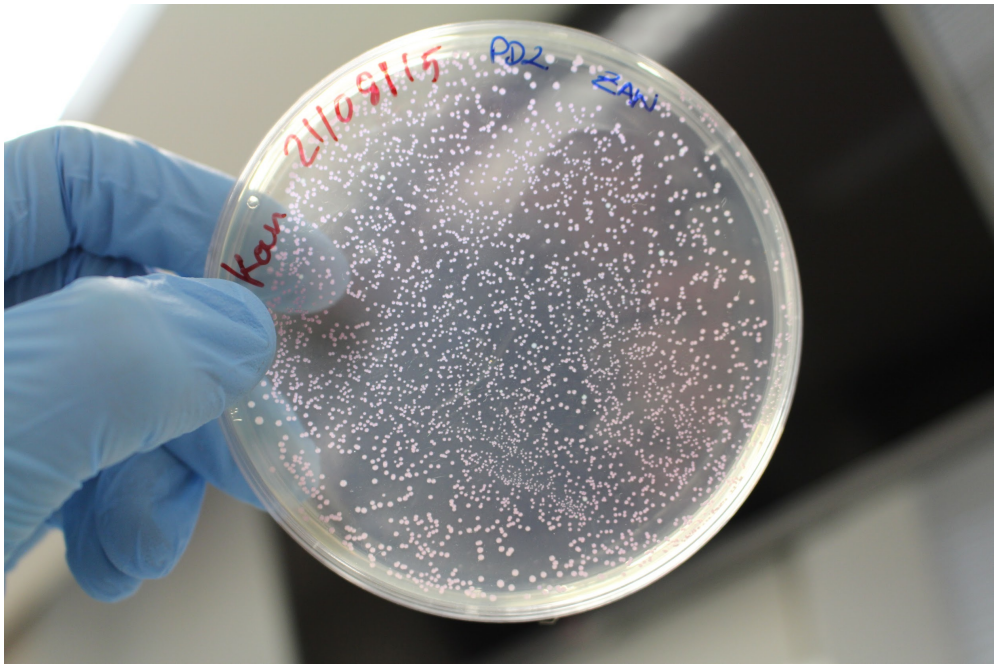

21/8/15
Carb +Cm +Kan
06/09/15
w/e ACRH1 + pD3

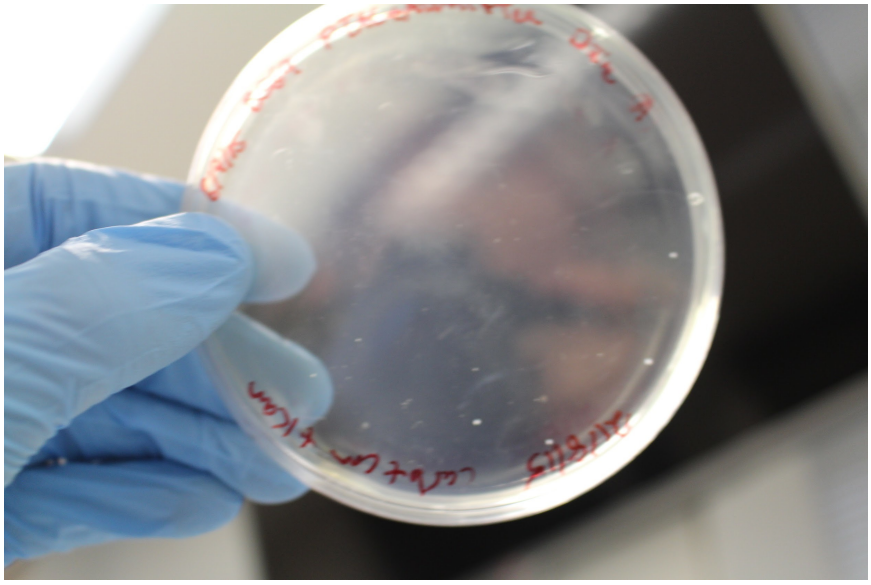

CM+Carb+kan
JOB
B568
+pD2
ACDH1

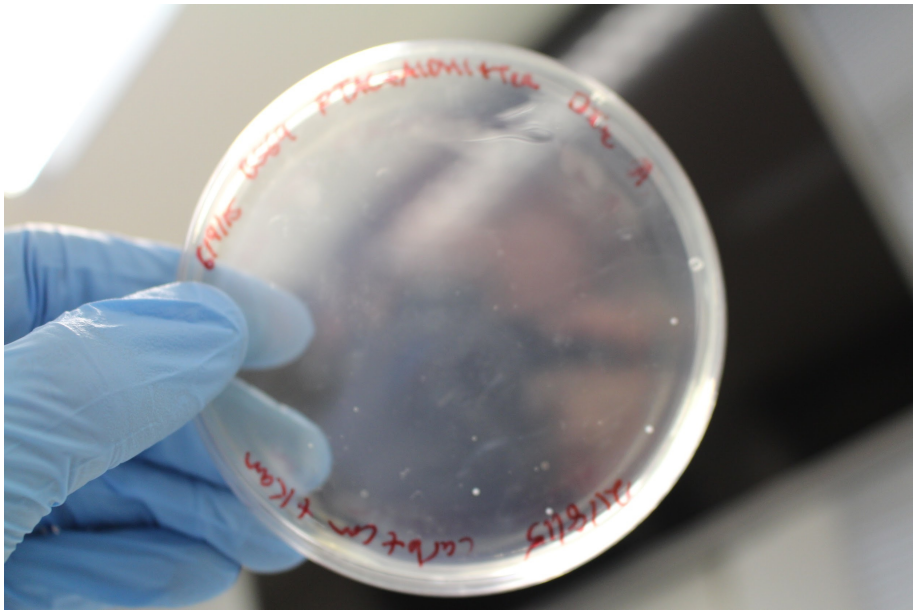

CM+kan
B568
pD1
ALDH1

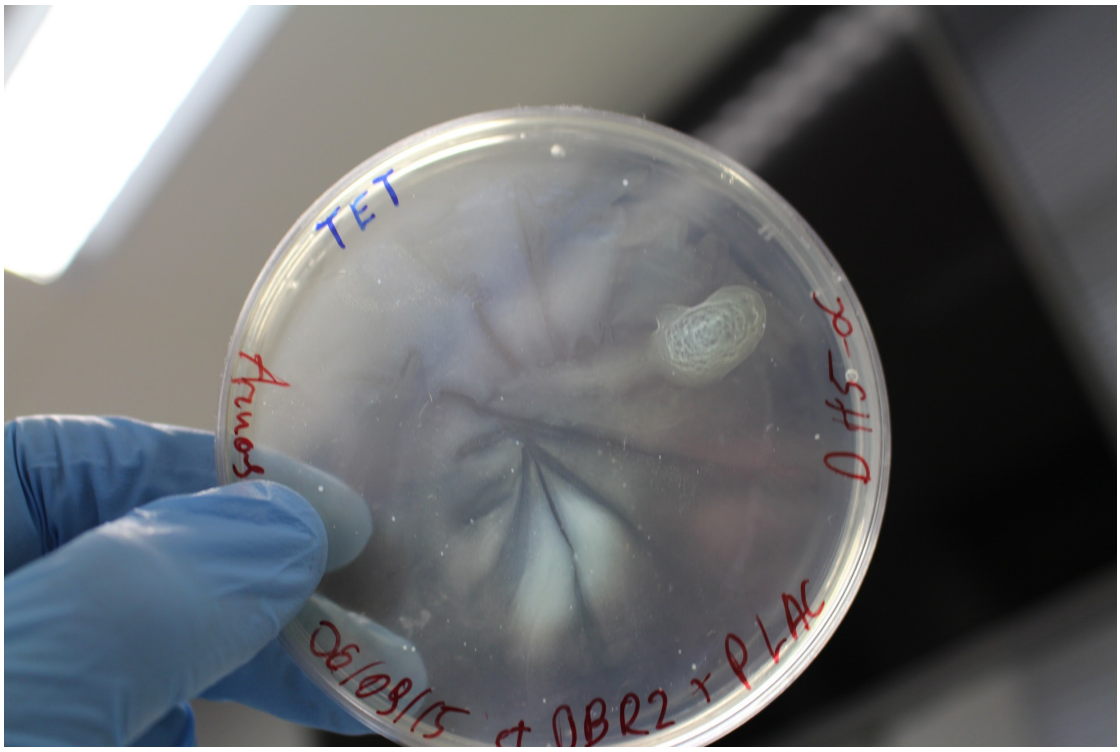

06/09/15
Carb
DH5-α
Amps 06/09/15
Δ DBR2 + PTA

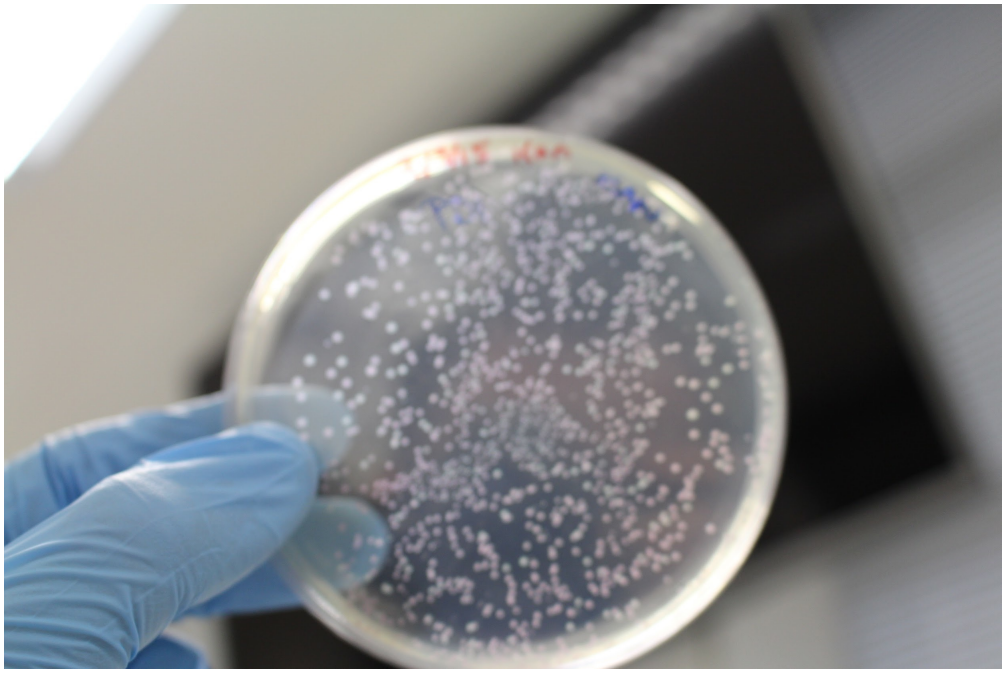

06/09/15
Carb
DH5-α
Δ DBR2 + PD3

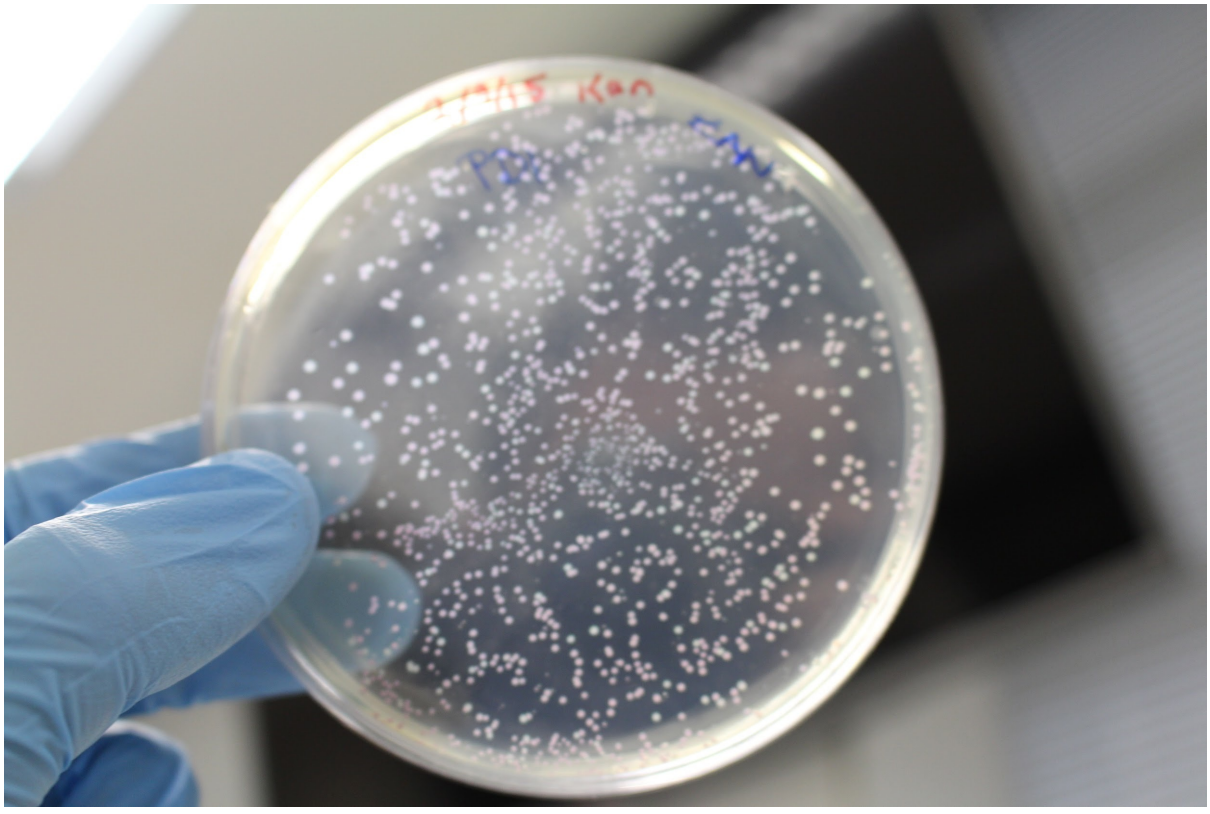

21/09/15
PD2
ZAN
Kan

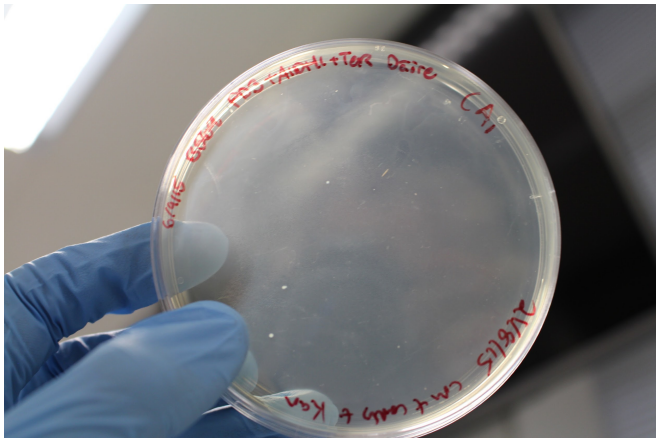


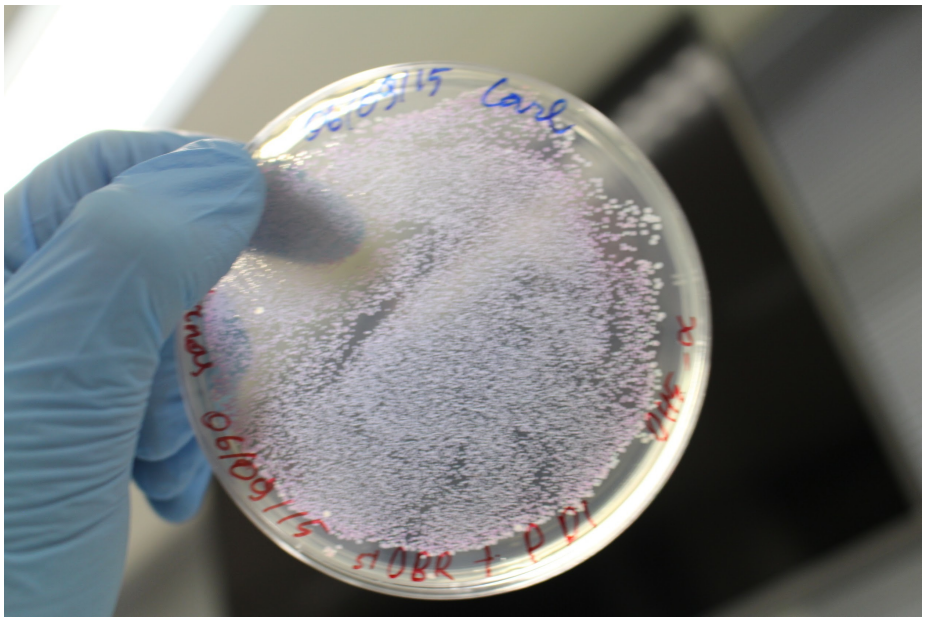


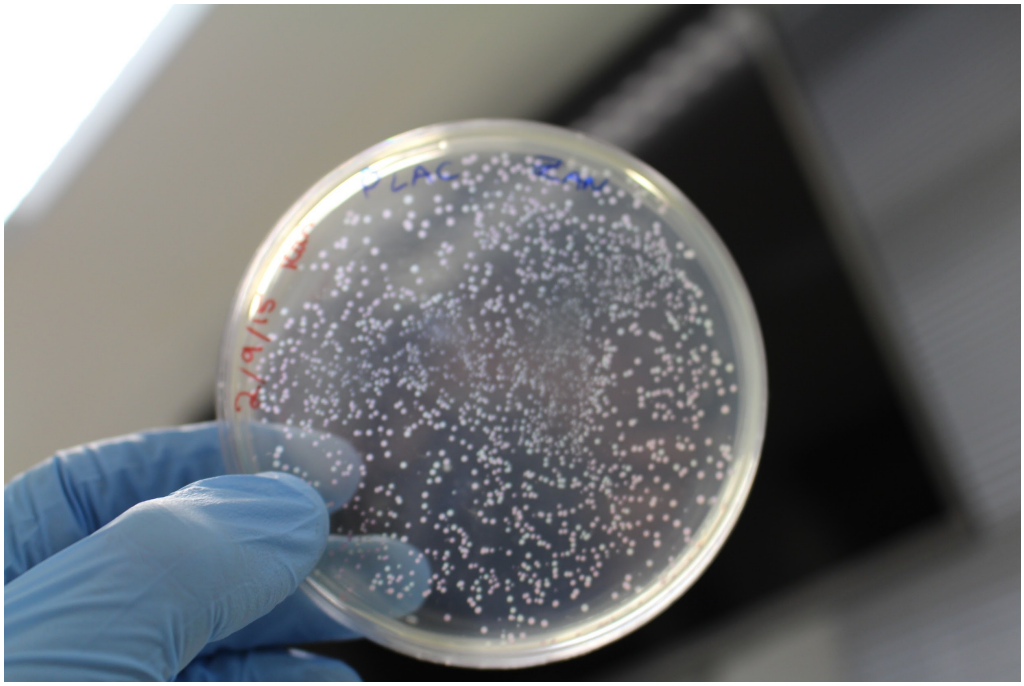

TET
06/09/15

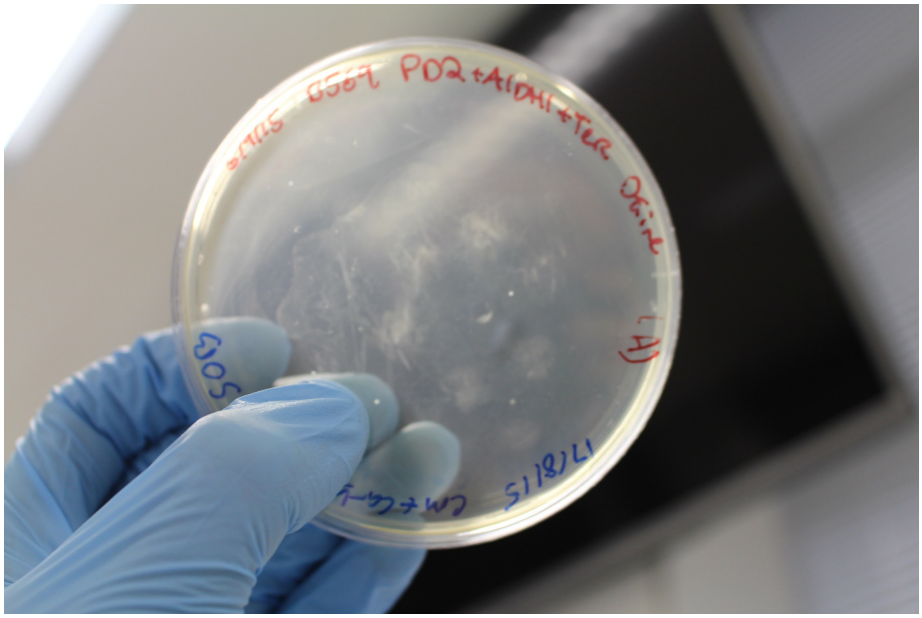


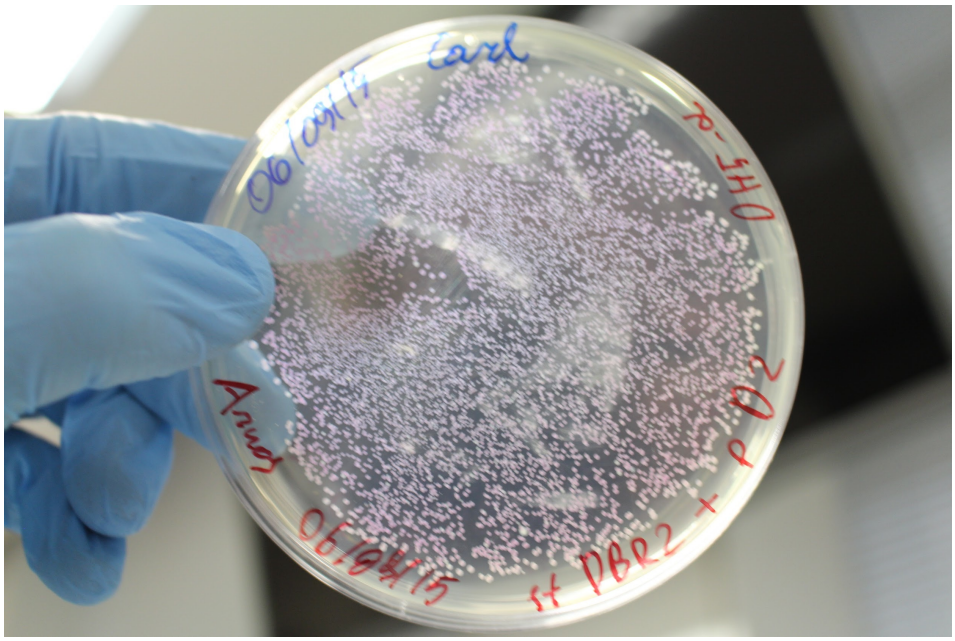


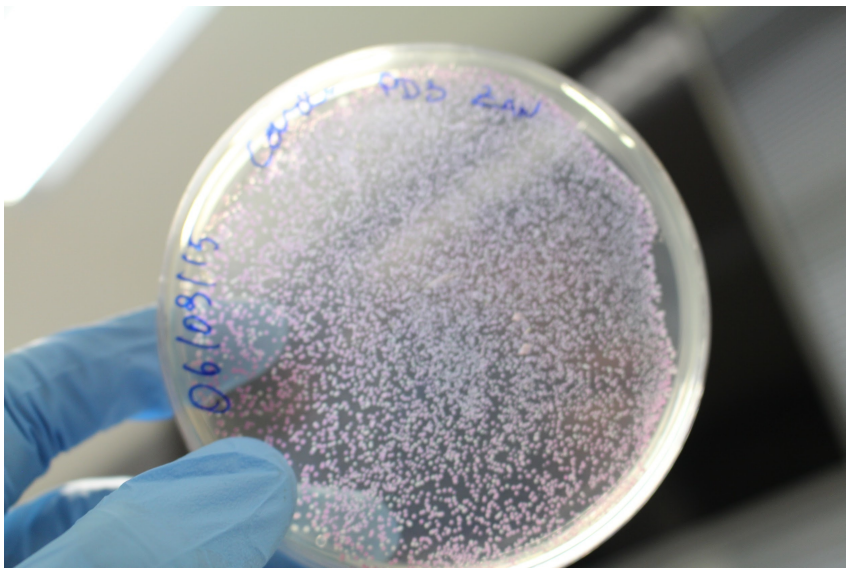


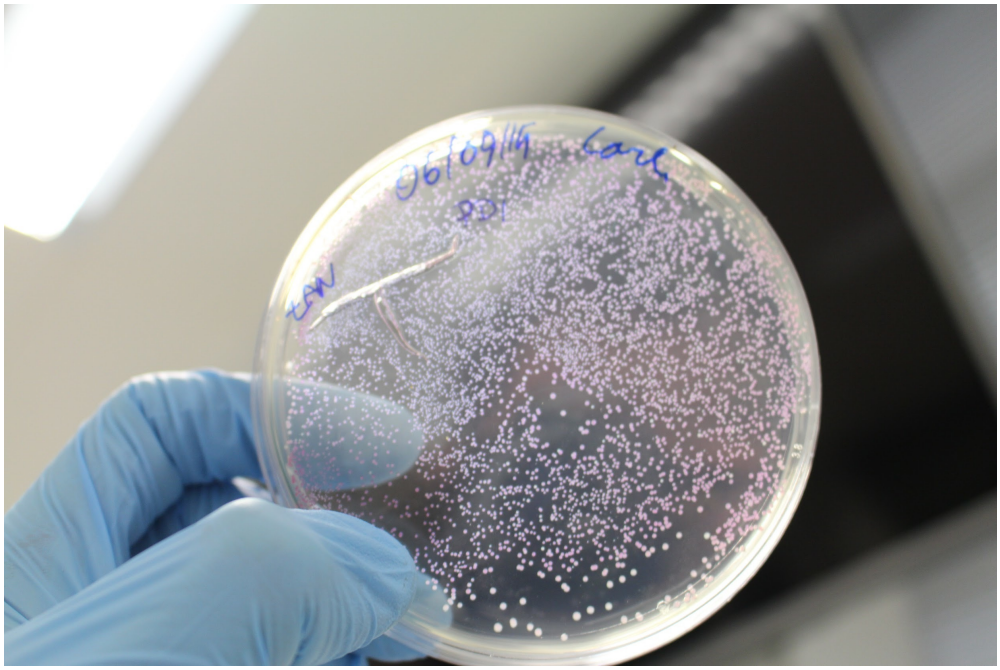


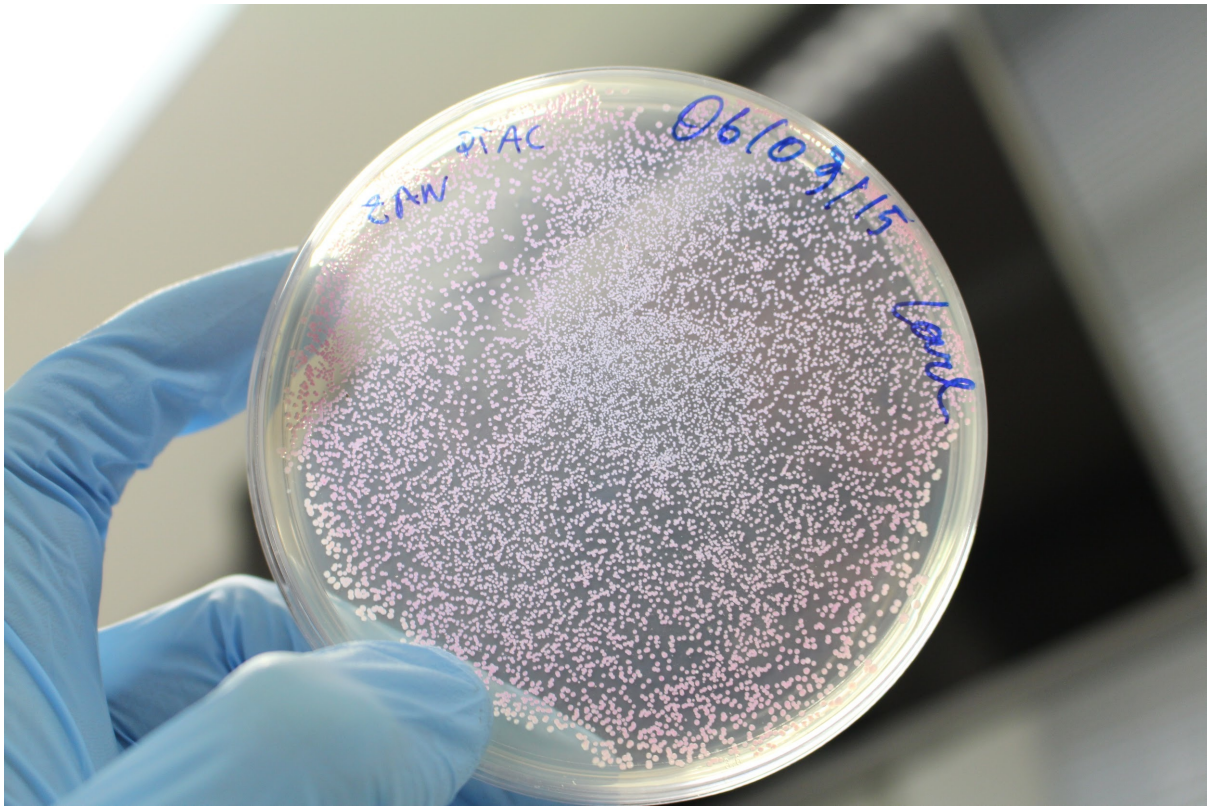


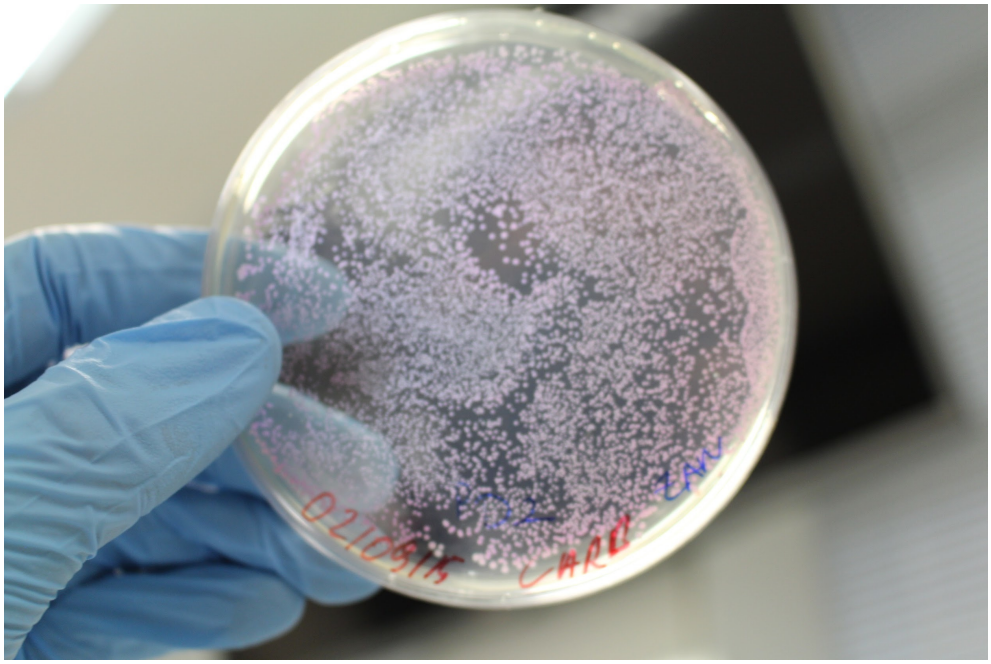


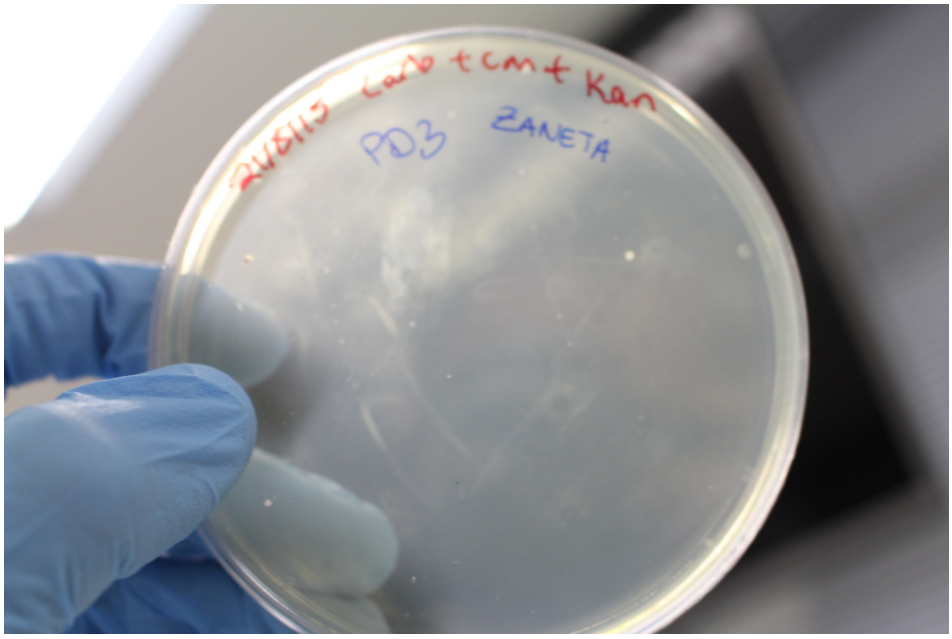


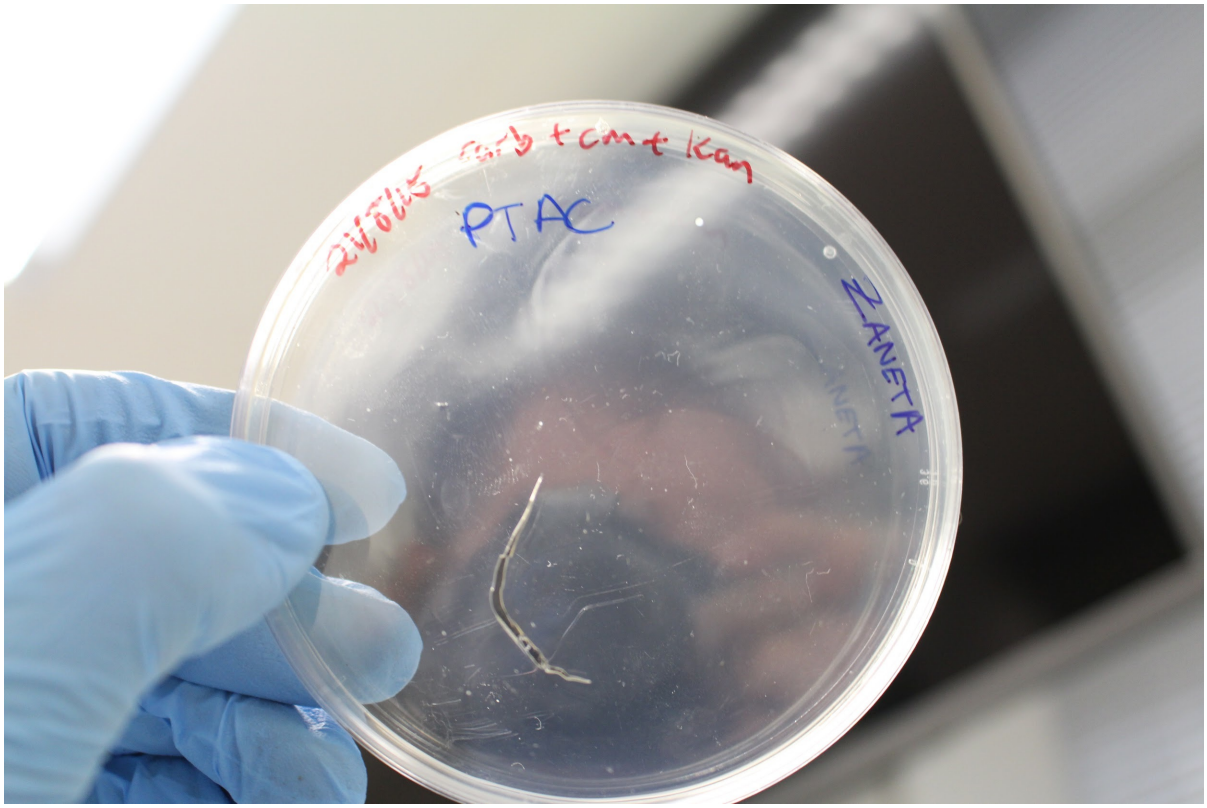


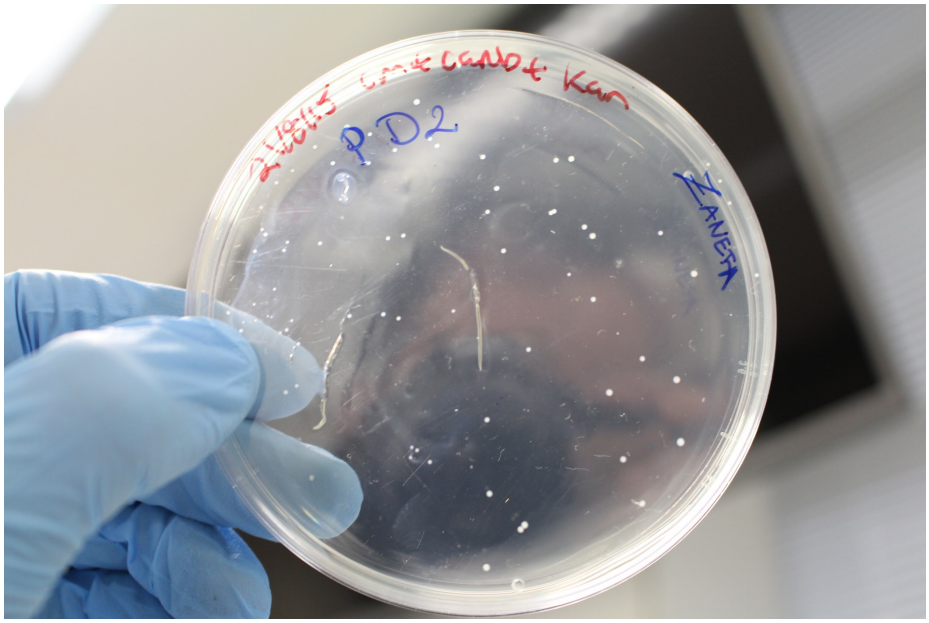

06/09/15
Lark
RDI
RAW

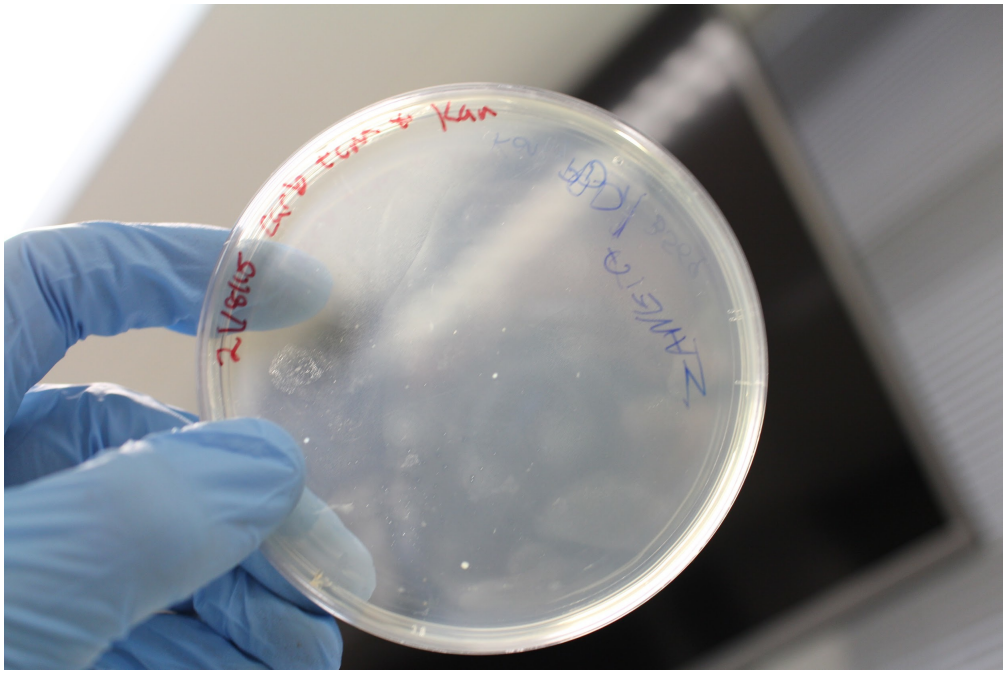

RAW
RTAC
06/09/15
Lark

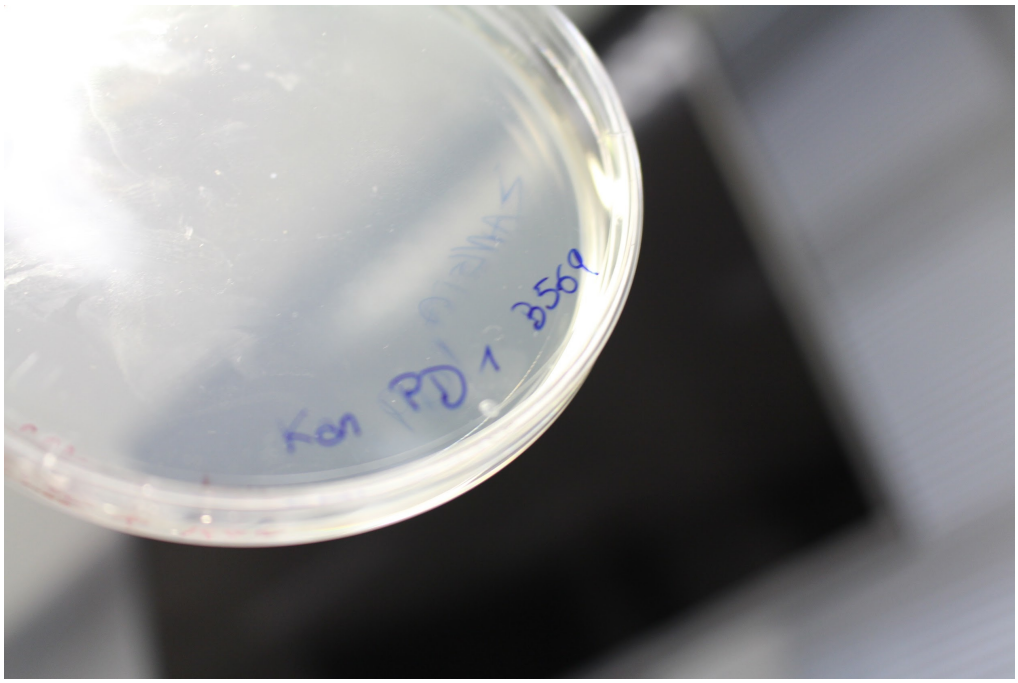

021015
CARB
KAN

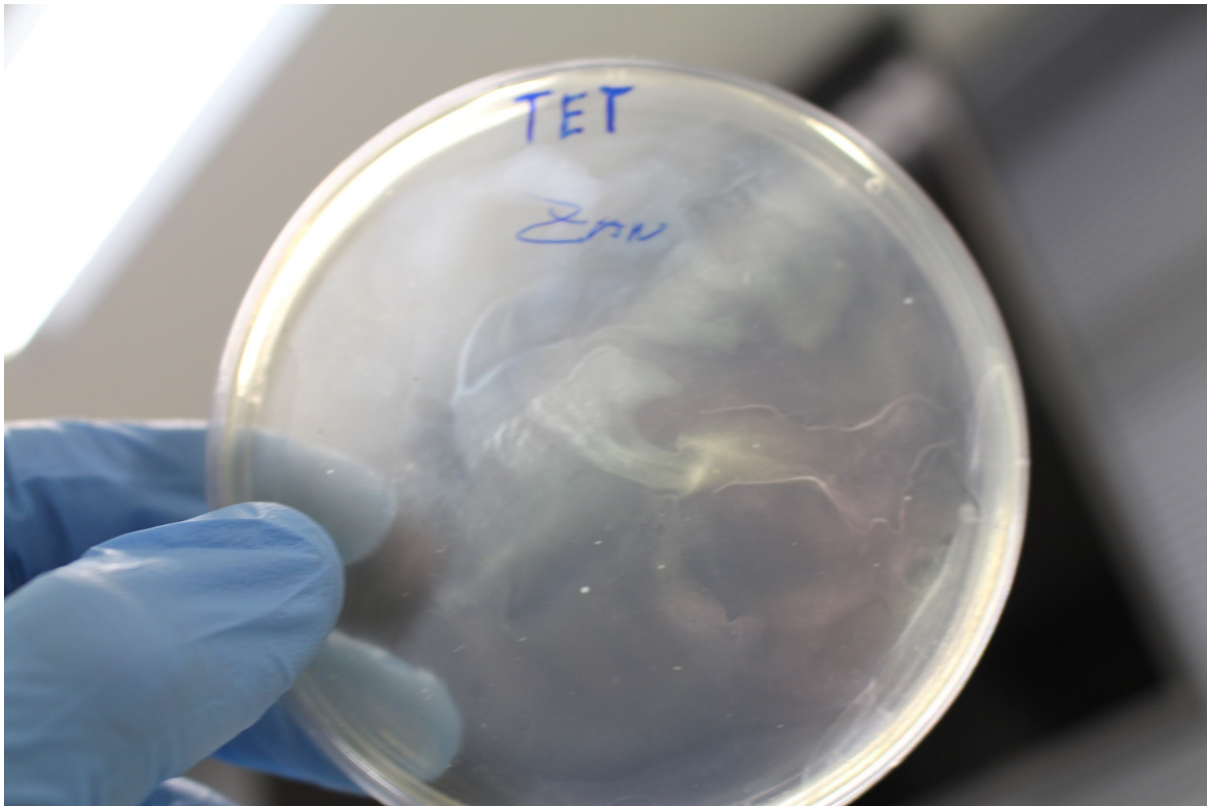

carb tcm t Kan
PD3
ZANETA

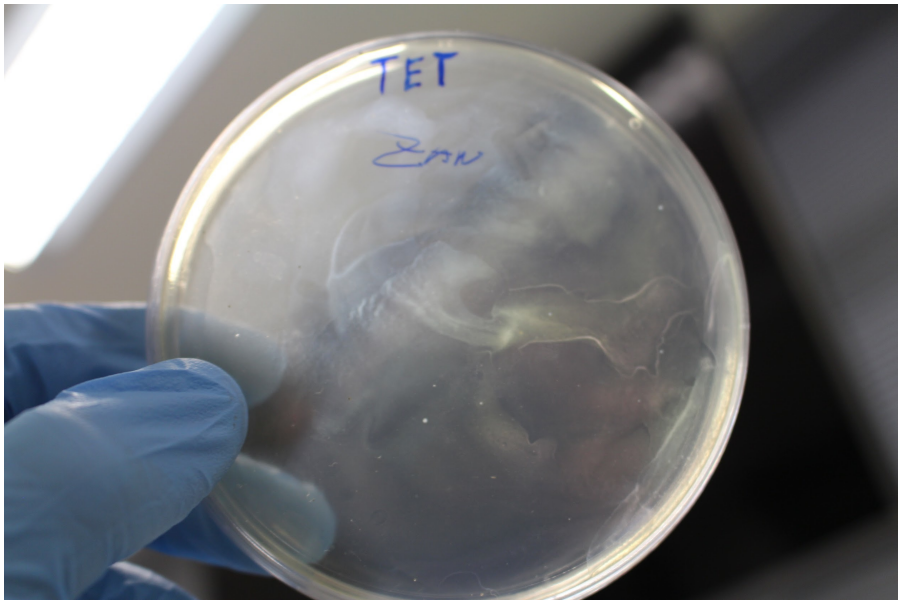

PTAC
ZANETA

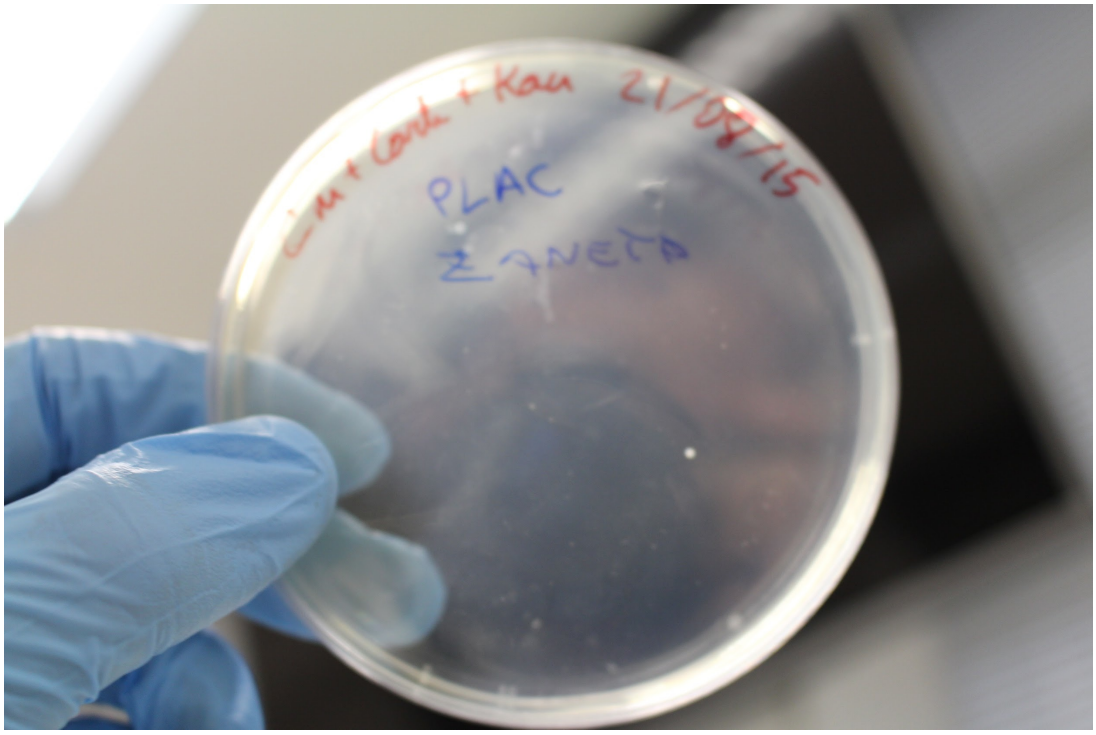

PD2
ZANETA

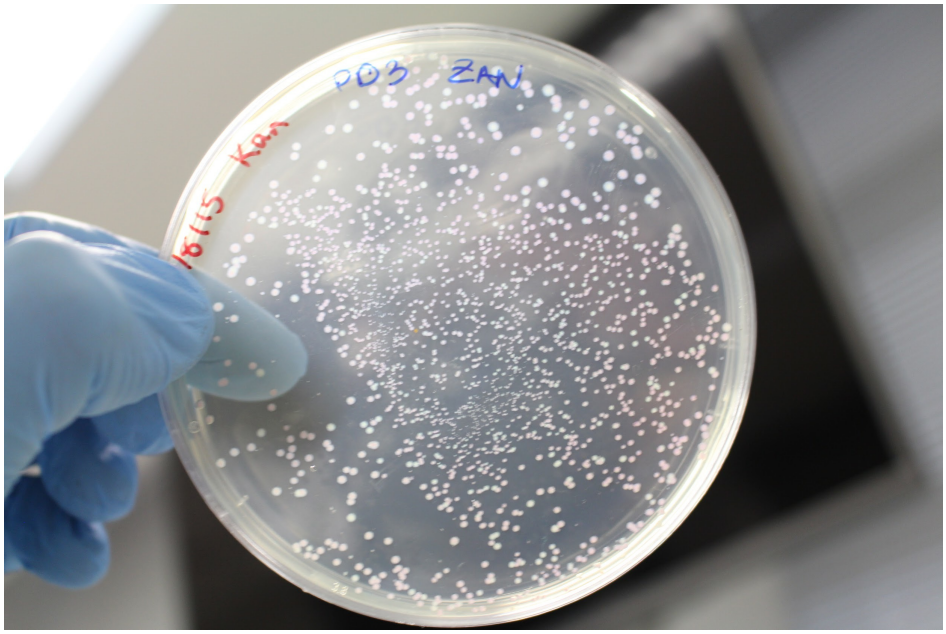

Kan


Kan
3569


TET


TET





Preparation of 400mL of LB medium 7th September:

Requirements:

- Balance
- Spatula
- Weighing Boat
- 500mL Glass Bottle
- 4g Sodium Chloride
- 4g Tryptone Enzymatic Digest from Casein
- 2g Yeast Extract
- 400mL dH2O
- Autoclave Tape
- Permanent Marker
- Aluminium foil
- Access to autoclave

Procedure:

Add the following to a labelled glass bottle:

- 4g Sodium Chloride
- 4g Tryptone Enzymatic Digest from Casein
- 2g Yeast Extract

- Dissolve in 350ml dH2O
- Make up solution to 400mL
- Loosen the cap of the glass bottle
- Cover the cap in aluminium foil
- Seal foil using autoclave tape
- Autoclave at 120^{o}C for 20 minutes

Preparation of 100mL of SOB medium: (Based on http://parts.igem.org/SOB) 7th September

Requirements:

- Balance
- Spatula
- Weighing Boat
- 300mL Glass Bottle
- 0.0584g Sodium Chloride
- 2g Tryptone Enzymatic Digest from Casein
- 0.5g Yeast Extract
- 100mL dH2O
- Autoclave Tape
- Permanent Marker
- Aluminium foil
- Access to autoclave

Procedure:

Add the following to a labelled glass bottle:

- 0.0584g Sodium Chloride
- 2g Tryptone Enzymatic Digest from Casein
- 0.5g Yeast Extract
- 0.0186g KCl
- 0.493g $MgSO_4$

- Dissolve in ~80ml dH2O
- Make up solution to 100mL
- Loosen the cap of the glass bottle
- Cover the cap in aluminium foil
- Seal foil using autoclave tape
- Autoclave at 120^{o}C for 20 minutes

Preparation of Broths from Transformation Plates 7th September:

Objective: To prepare broths of colonies growing on transformation plates.

Requirements:

- Colonies on transformation plates
- LB medium
- Appropriate antibiotic
- Loop
- Bunsen burner
- Tubes

Procedure: (Carry out work beside a lighted bunsen burner) (Select three colonies from each set of triplicate transformations. Example: choose three colonies from the three PD1+RBS+ALDH1+Terminator plates)

- Add 5mL of LB medium to a falcon tube.
- Add 5µL of the appropriate 1000x antibiotic. (In the case of B569 cells add 5µL of carbenicillin, chloramphenicol and kanamycin)
- Use a sterile loop to scoop up a colony from the plate (choose white colonies as red colonies are expressing RFP meaning that the RFP gene was re-ligated to the backbone rather than the backbone ligating to the desired device. B569 colonies all white as they have to be induced to express RFP).
- Inoculate the LB medium in the falcon tube by placing the loop into the tube and stirring.
- Sterilise the loop by putting it into the bunsen burner's flame before repeating the process for the desired amount of broths.
- Incubate broths at 37^{o}C with shaking overnight.

Miniprep 8th September: (identical protocol to that carried out on 3rd September)

Preparation of a 2% Agarose Gel 8th September: (identical protocol to that carried out on 3rd September)

Polymerase Chain Reaction (PCR) 8th September:

Requirements:

- Q5® High-Fidelity 2X Master Mix
- Forward and Reverse Primers (primers came dry from Sigma)
- TE Buffer / Nuclease free H2O
- Ice box
- 96 well-plate
- Thermocycler
- Pipettes, pipette tips

Objective: To determine if purified plasmid DNA contains the desired part. (.ie if RBS + ALDH1+Terminator is ligated to any of the promoters D1, D2, D3, PTAC, PLAC in DH5alpha and also in B569 and; if any of the successfully miniprepped RBS + DBR2 samples had promoter D1 or D2.

Procedure (Based on NEB Protocol for Q5® High-Fidelity 2X Master Mix)

- Design primers in such a way as to determine by PCR product size if the previous ligation was carried out correctly.
- Get primers synthesised.
- Make a PCR master mix for each primer pair:

- **RBS (w) +ALDH1 LP and RP** (To be run on 2% agarose gel)

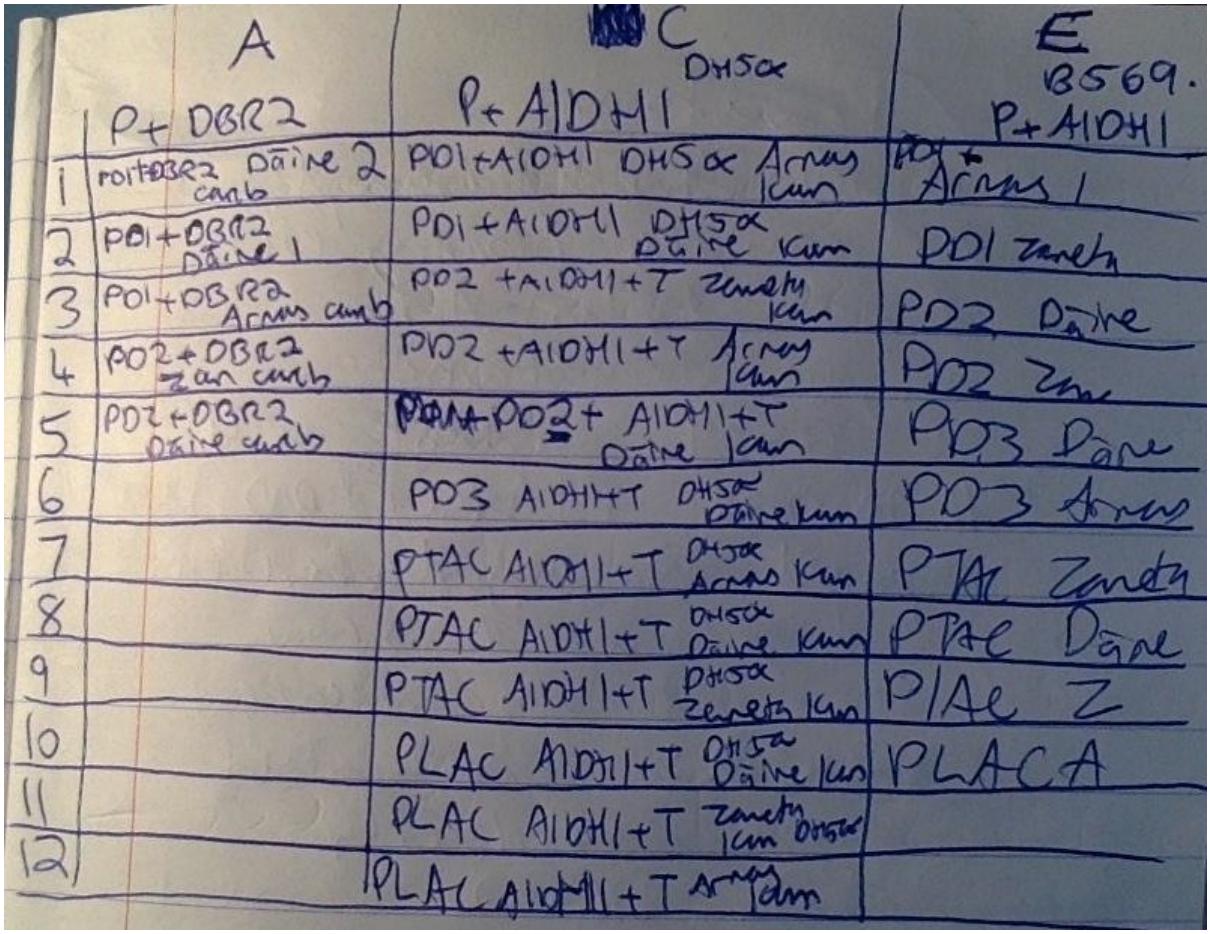
(Primers designed so that if promoter is present PCR products will be distinguishable in length to samples where the promoter isn't present.) There are 22 miniprepped samples between the DH5alpha and B569 samples. Create master mix for 24 samples to allow for pipetting error. Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:

- ❏ 300µL Q5® High-Fidelity 2X Master Mix
- ❏ 30µL of 10µM (add 10µL of 100µM forward primer to 90µL of TE Buffer in a separate tube) forward primer
- ❏ 30µL of 10µM reverse primer
- ❏ 192µL TE Buffer

- **PTAC+DBR2 LP and PTAC+DBR2 RP:** (To be run on 2% agarose gel)

(Primers designed so that if any of the promoters had been successfully ligated to the RBS +DBR2 the PCR products will be distinguishable in length to samples where the promoter isn't present.) There are five miniprepped samples. Create master mix for seven samples to allow for pipetting error. Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:

- ❏ 87.5µLµL Q5® High-Fidelity 2X Master Mix
- ❏ 8.75µL of 10µM (add 1µL of 100µM forward primer to 9µL of TE Buffer in a separate tube) forward primer
- ❏ 8.75µL of 10µM reverse primer
- ❏ 56µL TE Buffer



- Add 23µL of the appropriate master mix to pre-planned wells of the 96 well-plate.
- Add 2µL of template DNA to its corresponding well.
- Seal the well-plate.
- Transfer the well plate to the thermocycler.
- Use the NEB Tm Calculator to determine the annealing temperature.
- Set the program on the thermocycler:

❏ Initial Denaturation: 98°C for 30 seconds
❏ 32 cycles: 98°C for 5-10 seconds; 62°C for 10-30 seconds; 72°C for 20-30 seconds.
❏ Final Extension: 72°C for 2 minutes.

Transformation of Promoter + RBS + ALDH1 + Terminator purified samples miniprepped from DH5alpha into B569 competent cells 8th September: (based on NEB High Efficiency Transformation protocol)

Objective: To transform B569 cells with purified Promoter + RBS + ALDH1 + Terminator plasmid DNA from DH5alpha cells.

Requirements:

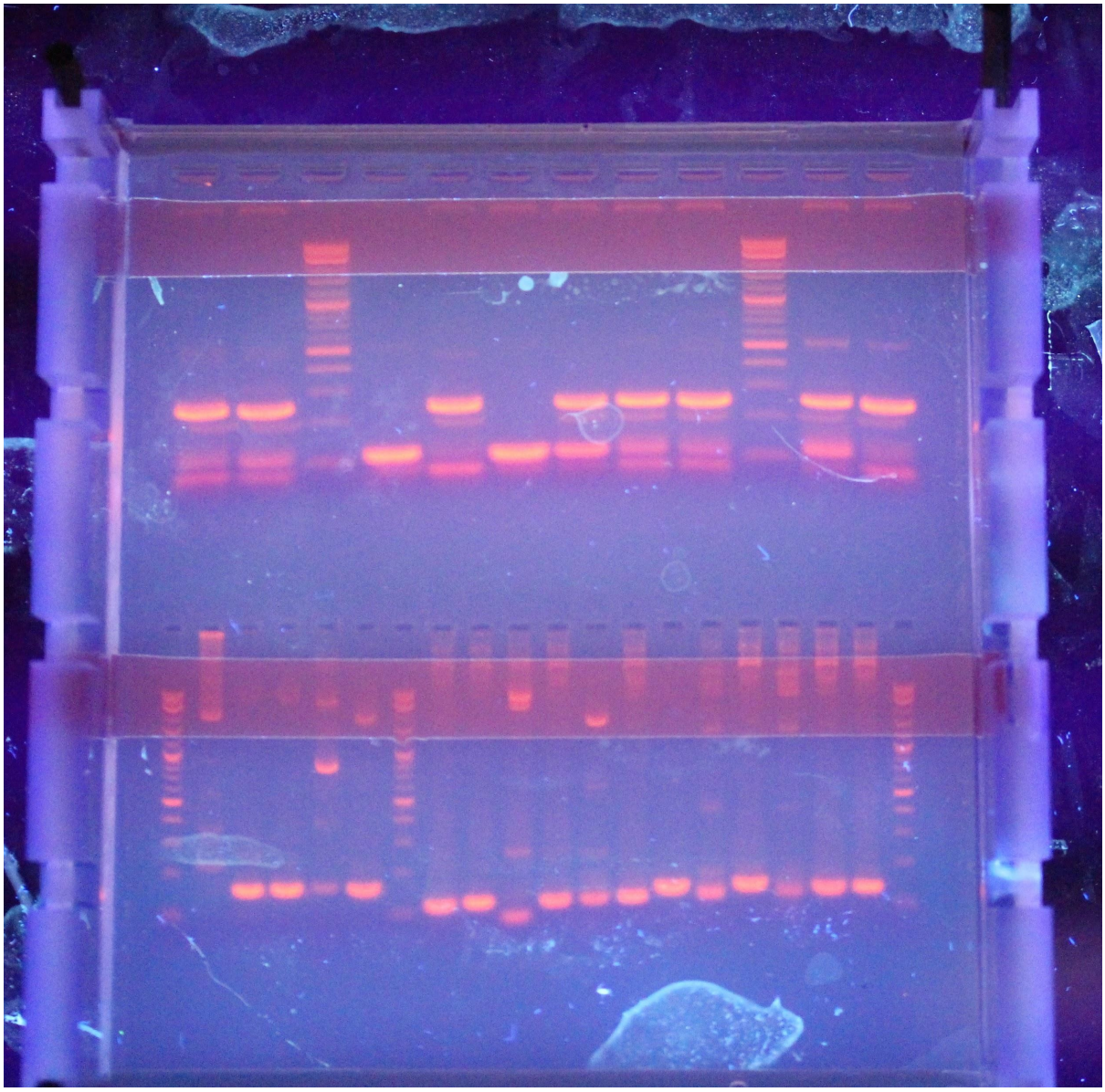
- 50µL of competent cells per transformation
- purified DNA
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42^{o}C
- Ice box
- Incubator
- 950µL of SOB medium per transformation
- eppendorf tubes
- bunsen burner
- Glass spreader
- Ethanol

Procedure:

- Pre-chill eppendorf tubes on ice.
- Thaw competent cells on ice.
- Add 50µL of B569 competent cells to each of the pre-chilled transformation tubes.
- Add 2µL of miniprepped device to its appropriate transformation tube ie. PD1+RBS +ALDH1+Terminator Arnas; PD1+RBS+ALDH1+Terminator Dáire; PD2+RBS +ALDH1+Terminator Zaneta; PD2+RBS+ALDH1+Terminator Arnas; PD2+RBS +ALDH1+Terminator Dáire; PD3+RBS+ALDH1+Terminator Dáire; PTAC+RBS +ALDH1+Terminator Arnas; PTAC+RBS+ALDH1+Terminator Dáire; PTAC+RBS +ALDH1+Terminator Zaneta; PLAC+RBS+ALDH1+Terminator Dáire; PLAC+RBS +ALDH1+Terminator Zaneta and PLAC+RBS+ALDH1+Terminator Arnas (transform all samples if gel hasn't been run, transform specifically chosen samples based on gel if it's run).
- Carefully flick the transformation tubes 4-5 times to mix the cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes.
- Heat-shock at 42^{o}C in the heat-block for exactly 30 seconds.
- Place the transformations on ice for 5 minutes.
- Pipette 950µL of SOB medium into each transformation tube beside a lighted bunsen burner.
- Incubate the transformations at 37^{o}C for 2 hours with shaking.
- Warm selection plates to 37^{o}C. Use carbenicillin, chloramphenicol and kanamycin triple antibiotic plates for ALDH1 device transformation into B569 cells (B569 strain originally has two plasmids, giving resistance to carbenicillin and chloramphenicol).
- 200µL of each tube was pipetted and plated.
- Pass the glass spreader through the bunsen burner's flame before spreading.
- Cool the glass spreader on part of the plate that does not contain cells before spreading the cells on the plate.
- Incubate plates overnight at 37^{o}C.

Running PCR products on a 2% agarose gels 8th September: (protocol was identical to that carried out on the 5th of September except that samples were only run on a 2% gel not both a 1% gel and a 2% gel.

Results:



2% agarose gel with products from primers RBS (w) +ALDH1 LP and RP and PTAC+DBR2 LP and PTAC+DBR2 RP to determine if promoters had successfully ligated to ALDH1 construct and the DBR2 construct.

Top Row: (B569)
Lane 1: PD1+RBS+ALDH1+Terminator Arnas
Lane 2: PD1+RBS+ALDH1+Terminator Zaneta
Lane 3: DNA Ladder
Lane 4: PD2+RBS+ALDH1+Terminator Dáire
Lane 5: PD2+RBS+ALDH1+Terminator Zaneta
Lane 6: PD3+RBS+ALDH1+Terminator Dáire
Lane 7: PD3+RBS+ALDH1+Terminator Arnas
Lane 8: PTAC+RBS+ALDH1+Terminator Zaneta
Lane 9: PTAC+RBS+ALDH1+Terminator Dáire
Lane 10: DNA Ladder
Lane 11:PLAC+RBS+ALDH1+Terminator Zaneta
Lane 12: PLAC+RBS+ALDH1+Terminator Arnas

Bottom Row: (DH5alpha)
Lane 1: DNA Ladder
Lane 2: PD1+RBS+DBR2 Dáire 2
Lane 3 PDI+RBS+DBR2 Dáire 1
Lane 4: PD1+RBS+DBR2 Arnas
Lane 5: PD2+RBS+DBR2 Zaneta
Lane 6: PD2+RBS+DBR2 Dáire
Lane 7: DNA Ladder

Lane 8: PD1+RBS+ALDH1+Terminator Arnas
Lane 9: PD1+RBS+ALDH1+Terminator Dáire
Lane 10: PD2+RBS+ALDH1+Terminator Zaneta
Lane 11: PD2+RBS+ALDH1+Terminator Arnas
Lane 12: PD2+RBS+ALDH1+Terminator Dáire
Lane 13: PD3+RBS+ALDH1+Terminator Dáire
Lane 14: PTAC+RBS+ALDH1+Terminator Arnas
Lane 15: PTAC+RBS+ALDH1+Terminator Dáire
Lane 16: PTAC+RBS+ALDH1+Terminator Zaneta
Lane 17: PLAC+RBS+ALDH1+Terminator Dáire
Lane 18: PLAC+RBS+ALDH1+Terminator Zaneta
Lane 19: PLAC+RBS+ALDH1+Terminator Arnas
Lane 20: DNA Ladder

Conclusions:
Lane 4 (PD2+RBS+ALDH1+Terminator Dáire) and 6 (PD3+RBS+ALDH1+Terminator Dáire) of the B569 samples showed clear bands of the expected length and so were chosen to be inoculated directly into cultures to run in the fermenter.

There were clear bands of expected length in the gel of (bottom row DH5alpha) Lane 8: PD1+RBS+ALDH1+Terminator Arnas and Lane 14: PTAC+RBS+ALDH1+Terminator Arnas. Based on this, 800µL of the transformation broth of these plasmids into B569 were inoculated into cultures to be run in the fermenter.

The bottom row lane 3 (PDI+RBS+DBR2 Dáire 1), 4 (PD1+RBS+DBR2 Arnas) and 6 (PD2+RBS+DBR2 Dáire) showed clear bands of expected length. It was decided to use these samples for ligation to RBS+ALDH1+Terminator. (mistakenly PD2+RBS+DBR2 Zaneta lane 5 was used instead of lane 6 PD2+RBS+DBR2 Dáire).

Sent samples for sequencing 9th September:

Minipreps from B569:
- PD2 + RBS + ALDH1 + T D (Broth had been inoculated into culture)
- PD3 + RBS + ALDH1 + T D (Broth had been inoculated into culture)
- PD2 + RBS + ALDH1 + T Z
- PD3 + RBS + ALDH1 + T A

Minipreps from DH5alpha:

- PD2 + RBS + ALDH1 + T Zaneta
- PD1 + RBS + ALDH1 + T Arnas (miniprep from DH5alpha was transformed into B569 and transformation SOB was inoculated directly into culture)
- PTAC + RBS + ALDH1 + T Arnas (miniprep from DH5alpha was transformed into B569 and transformation SOB was inoculated directly into culture)
- PD1 + RBS + DBR2 Dáire 1
- PD1 + RBS + DBR2 Arnas
- PD2 + RBS + DBR2 Zaneta

Digestion and Ligation 9th September:

Objective: To ligate Promoter+RBS+DBR2 samples to RBS+ALDH1+Terminator samples.

Requirements:

- NEBuffer 2.1
- Eco-RI HF®
- PstI
- SpeI
- XbaI
- dH2O
- Pipettes, pipette tips
- Water bath and/or heat block/thermocycler
- eppendorf tubes
- permanent marker
- T4 DNA ligase reaction buffer
- T4 DNA ligase
- ice box

Procedure (based on iGEM protocol http://parts.igem.org/Help:Protocols/3A_Assembly)

- Calculate the required amount of enzyme master mixes.

Prepare Enzyme master mix for plasmid backbone by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL PstI
- 18.5µL dH2O

Prepare Enzyme master mix for Part A by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL SpeI
- 18.5µL dH2O

Prepare Enzyme master mix for Part B by adding the following to an eppendorf tube:

- 5.5µL NEBuffer 2.1
- 0.5µL PstI
- 0.5µL XbaI
- 18.5µL dH2O

Digestion:

Digest RFP kanamycin (BBa_J04450) (conc. 167.5 ng/µL) by adding the following to a labelled eppendorf tube:

- 0.6µL RFP kanamycin
- 3.4µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest PD1+RBS+DBR2 Dáire 1 (in ampicillin backbone) (conc. 53.1 ng/µL) by adding the following to a labelled eppendorf tube:

- 1.88µL PD1+RBS+DBR2 Dáire 1
- 2.11µL dH2O
- 4µL enzyme master mix for part A

Digest PD1+RBS+DBR2 Arnas (in ampicillin backbone) (conc. 53.1 ng/µL) by adding the following to a labelled eppendorf tube:

- 1.88µL PD1+RBS+DBR2 Arnas
- 2.11µL dH2O
- 4µL enzyme master mix for part A

Digest PD2+RBS+DBR2 Zaneta (in ampicillin backbone) (conc. 80.5 ng/µL) by adding the following to a labelled eppendorf tube:

- 1.24µL PD2+RBS+DBR2 Zaneta
- 2.76µL dH2O
- 4µL enzyme master mix for part A

Digest RBS+ALDH1+T 1// (in ampicillin backbone) (conc. 82.3 ng/µL) by adding the following to a labelled eppendorf tube:

- 1.22µL RBS+ALDH1+T 1//
- 2.78µL dH2O
- 4µL enzyme master mix for part A

Incubate the digestions at 37^{o}C for 30 minutes. Heat-kill at 80^{o}C for 20 minutes.

Ligation:

Ligate the appropriate Promoter+RBS+DBR2 to RBS+ALDH1+T 1// by adding the following to labelled eppendorf tubes:

- 1µL digested RFP kanamycin
- 2µL of the appropriate digested Promoter+RBS+DBR2
- 2µL of digested RBS+ALDH1+T 1//
- 1µL T4 DNA ligase reaction buffer
- 0.5µL T4 DNA ligase
- 3.5µL dH2O

Incubate the ligations at 16^{o}C for 30 minutes. Heat-kill at 80^{o}C for 20 minutes.

Transformation: (based on NEB High Efficiency Transformation protocol)

Objective: To transform the ligation products (.ie Promoter+RBS+DBR2 to RBS+ALDH1+T) into DH5alpha and B569 cells.

Requirements:

- 50µL of competent cells per transformation
- purified DNA
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42^{o}C
- Ice box
- Incubator
- 950µL of SOB medium per transformation
- eppendorf tubes
- bunsen burner
- Glass spreader
- Ethanol

- Pre-chill eppendorf tubes on ice.
- Thaw competent cells on ice.
- Add 50µL of competent cells to each of the pre-chilled transformation tubes.
- Add 2µL of ligation product to its appropriate transformation tube. (.ie PD1+RBS +DBR2 Dáire 1+RBS+ALDH1+Terminator 1//; PD1+RBS+DBR2 Arnas +RBS +ALDH1+Terminator 1// and PD2+RBS+DBR2 Zaneta +RBS+ALDH1+Terminator 1//)
- Carefully flick the transformation tubes 4-5 times to mix the cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes.
- Heat-shock at 42^{o}C in the heat-block for exactly 30 seconds.
- Place the transformations on ice for 5 minutes.
- Pipette 950µL of SOB medium into each transformation tube.
- Incubate the transformations at 37^{o}C for an hour with shaking.
- Warm selection plates to 37^{o}C. (Use kanamycin plates for DH5alpha transformations. Use carbenicillin, chloramphenicol and kanamycin triple antibiotic plates for device transformation into B569 cells (B569 strain originally has two plasmids, giving resistance to carbenicillin and chloramphenicol).
- Spin the cells at 5g for 2 minutes.
- Remove 800µL of the supernatant from each tube and discard.
- Aspirate a number of times to resuspend the pellet before plating what's left in the transformation tube.
- Pass the glass spreader through the bunsen burner's flame before spreading.
- Cool the glass spreader on part of the plate that does not contain cells before spreading the cells on the plate.
- Incubate plates overnight at 37^{o}C.

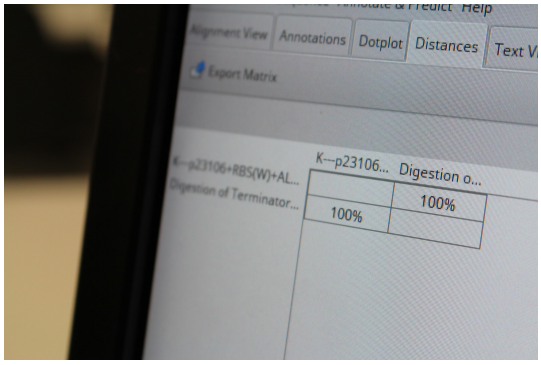
Sequencing Results 10th September:

Notable results:

PDI + RBS + DBR2 Arnas
Pairwise alignment (using Geneoius): 100% DBR2 and promoter

PDI + RBS + DBR2 Dáire 1
100 % DBR2 and promoter

PD1 + RBS + ALDH1 + Terminator Arnas
100% identity for promoter and terminator



Preparation of Broths 10th September:

Objective: To prepare broths from all of the transformation plates made the previous day. (ie. Promoter + RBS + DBR2 + RBS + ALDH1 + Terminator)

Requirements:

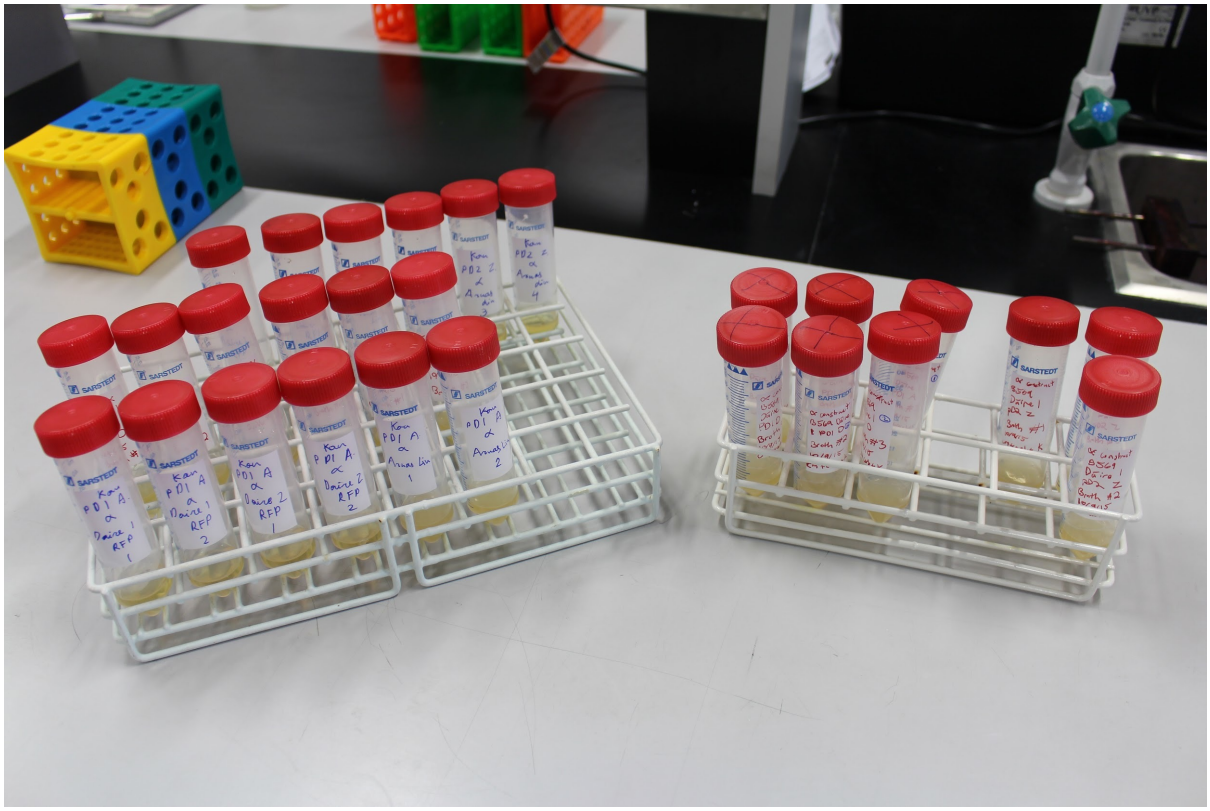
- 5mL LB medium per broth
- Appropriate antibiotics
- Tubes
- Incubator with shaker
- Transformation plates
- Wire loop
- Bunsen burner
- permanent marker

Procedure:

- Add 5mL of LB medium to a falcon tube.
- Add 5µL of the appropriate 1000x antibiotic. For DH5alpha samples add 5µL of kanamycin, In the case of the B569 samples add 5µL of carbenicillin, chloramphenicol and kanamycin.
- Use a sterile loop to scoop up a colony from the plate.
- Inoculate the LB medium in the falcon tube by placing the loop into the tube and stirring.

- Sterilise the loop by putting it into the bunsen burner's flame before repeating the process for the desired amount of broths.
- Incubate broths overnight at 37°C with shaking (inoculate three of the cloudiest samples of the construct in B569 into cultures in the fermentor. The tubes chosen were B569 Dáire 1 PD1 D Broth 1 and 2 along with B569 Dáire 1 PD1 A Broth 3.)

Results:



Miniprep 11th September: (followed same procedure as 5th September)

Preparation of a 1% Agarose Gel 11th September: (followed same procedure as 5th September)

Preparation of 300mL of LB Agar 11th September:

Objective: To prepare plates for selection.

Requirements:

- 3g NaCl
- 3g Tryptone Enzymatic Digest from Casein
- 1.5g Yeast Extract

- 4.5g Agar
- Glass bottle
- 300mL dH2O
- Spatula
- Balance
- Weighing boat
- Aluminium foil
- Autoclave tape
- Permanent marker
- Petri dishes
- Access to autoclave
- Flow cabinet / bunsen burner
- 1000x antibiotic solution
- P1000 pipette, pipette tips
- Parafilm

Procedure:

Add the following to a glass bottle:

- 3g NaCl
- 3g Tryptone Enzymatic Digest from Casein
- 1.5g Yeast Extract
- ~250mL of water

- Dissolve and make up to 300mL
- Add 4.5g Agar
- Loosen the cap of the glass bottle
- Cover the cap in aluminium foil
- Seal foil using autoclave tape
- Autoclave at 120^{o}C for 20 minutes
- Allow to cool until bottle is touchable
- In the flow cabinet: Add 300µL of 1000x antibiotic (kanamycin)
- Swirl the bottle to mix the antibiotic
- Pour plates
- Place the lid of the plate over half of the plate and leave for ~20 minutes to solidify
- Cover the plates with their lids
- Label plates and seal vents with parafilm
- Store plates at 4^{o}C

Polymerase Chain Reaction (PCR) 11th September:

Objective: To determine if purified plasmid DNA contains the desired device. ie. Promoter + RBS + DBR2 + RBS + ALDH1 + Terminator.

Requirements:
Q5® High-Fidelity 2X Master Mix

Forward and Reverse Primers
TE Buffer / Nuclease free H2O
eppendorf tubes
Ice box
96 well-plate
thermocycler
Pipettes, pipette tips

Procedure (Based on NEB Protocol for Q5® High-Fidelity 2X Master Mix)

- Make a PCR master mix for VF2 and VR primer pair:

- **VF2 and VR:** (To be run on a 1% agarose gel)

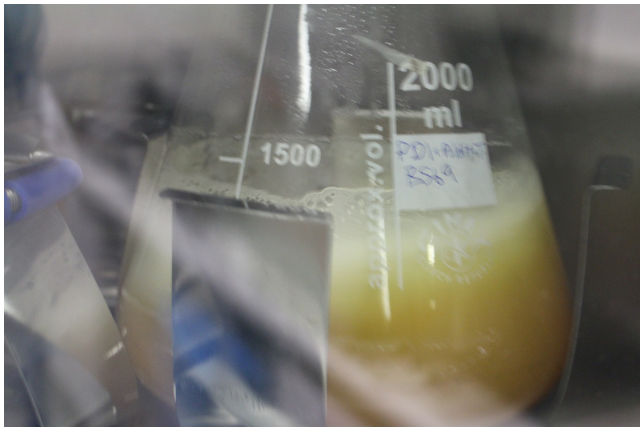
(Based on PCR product size it is hoped that it can be determined whether or not Promoter +RBS+DBR2 has successfully ligated to RBS+ALDH1+Terminator). There is a total of 13 miniprepped samples. Create master mix for 15 reactions to allow for pipetting error. Pre-chill master mix tube and keep on ice. For 25µL reactions add the following to the master mix tube:

- ❏ 187.5µL Q5® High-Fidelity 2X Master Mix
- ❏ 18.75µL of 10µM forward primer
- ❏ 18.75µL of 10µM reverse primer
- ❏ 120µL TE Buffer

- Add 23µL of the appropriate master mix to pre-planned wells of the 96 well-plate.
- Add 2µL of template DNA to its corresponding well. ie. B560 PD1 A alpha construct (Promoter + RBS + DBR2 + RBS + ALDH1) D1 1; B569 PD1 A alpha D1 2; B569 PD1 A alpha D1 3; B569 PD1 D alpha D1 1; B569 PD1 D alpha D1 2; B569 PD1 D alpha D1 3; PD1 D alpha D2 RFP 1; PD1 D alpha A linearized (lin) 1; PD1 D alpha A lin 2; PD1 D alpha D1 RFP 1; PD1 D alpha D1 RFP 2; PD1 A alpha A lin 1 and PD1 A alpha A lin 2.
- Seal the well-plate.
- Transfer the well plate to the thermocycler.
- Use the NEB Tm Calculator to determine the annealing temperature.
- Set the program on the thermocycler:
- ❏ Initial Denaturation: 98^{o}C for 30 seconds
- ❏ 32 cycles: 98^{o}C for 5-10 seconds; 62^{o}C for 10-30 seconds; 72^{p}C for 20-30 seconds.
- ❏ Final Extension: 72^{o}C for 2 minutes.

Taking a sample for GCMS analysis from the fermenter 11th September: (Repeated daily after IPTG induction)

Objective: To take of a sample of the culture in the fermenter to later test for the presence of artemisinic acid.



Procedure:

- Remove 25mL of culture from the conical flasks containing the constructs; PD1 Arnas + RBS + ALDH1 + Terminator and PTAC Arnas + RBS + ALDH1 + Terminator.
- Add 20mL of feed to each conical flask.
- Centrifuge the samples at 3000g for ten minutes.
- Remove the supernatant
- Add the minimum amount of water to resuspend
- Vortex
- Transfer sample to eppendorf
- Add small bit of water
- Vortex to resuspend what's left then add to the same corresponding eppendorf
- Centrifuge eppendorfs at 3000g for 10 minutes
- Remove all supernatant
- Add up to 0.5µL with water
- Resuspend what's left by vortexing

Running PCR products on a 1% agarose gel 11th September:

Objective: To investigate if PCR products are the desired size, to determine whether or not previous ligation of Promoter+RBS+DBR2 and RBS+ALDH1+Terminator had been successful.

Requirements:

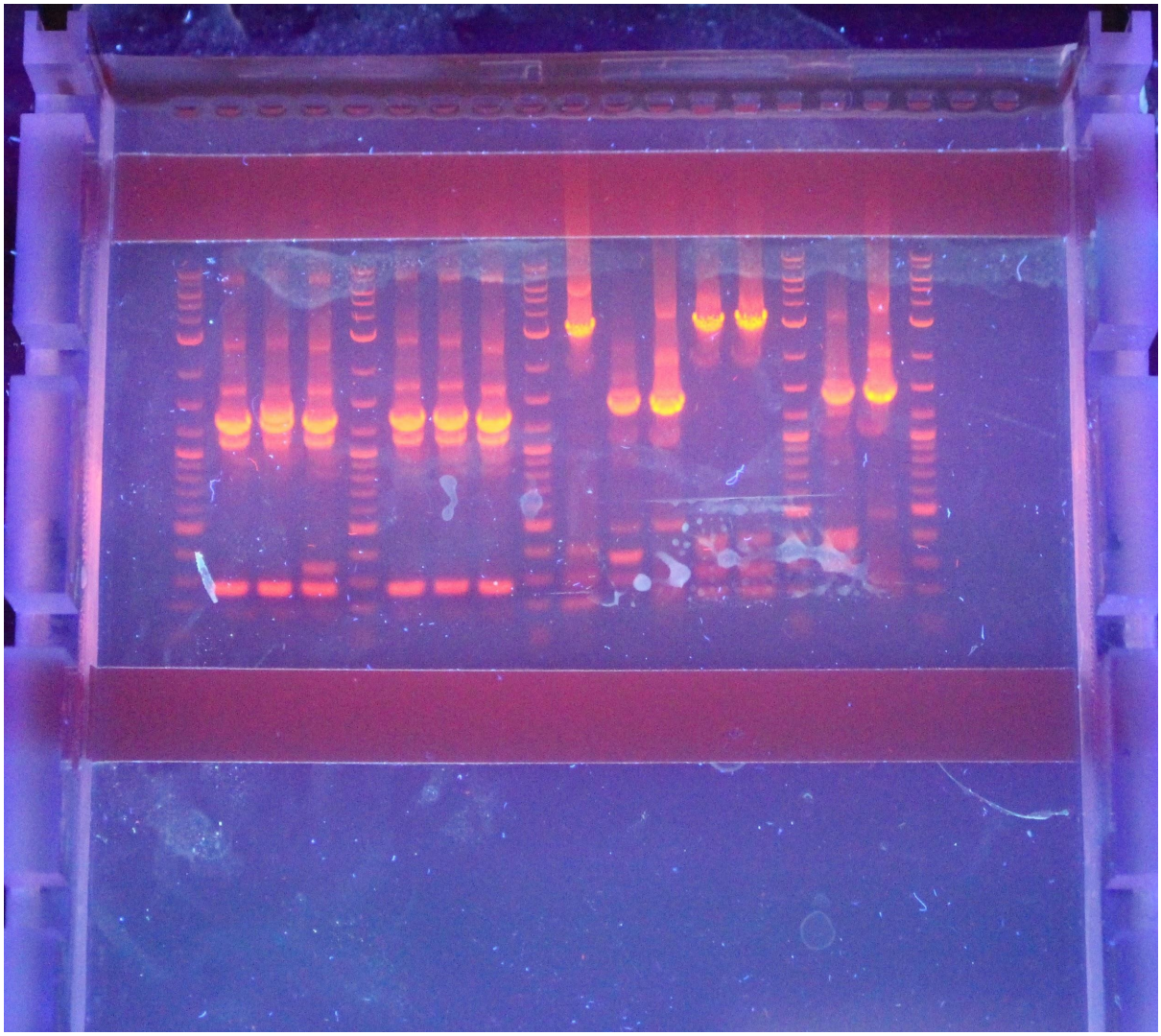
- 1% agarose gel
- 1x TBE buffer
- PCR products
- NEB Gel Loading Dye, Purple 6x
- NEB Quick-Load® Purple 2-Log DNA Ladder (0.1 - 10.0 kb)
- P20 calibrated pipette, pipette tips
- Electrodes
- Gel cover
- Power source
- UV light source

Procedure:

- Prepare a 1% agarose gel.

- When gel has set, position it correctly in the gel tray and remove the comb.
- Submerge the gel in 1x TBE buffer.
- Add 5µL of NEB Gel Loading Dye, Purple 6x to each PCR well containing a sample.
- Prepare a number of Quick-Load® Purple 2-Log DNA Ladder (0.1 - 10.0 kb) samples to act as a reference for size.
- Create a plan as to which lane each sample will run.
- Add 15µL of each sample to its appropriate lane in the gel.
- Place the cover on the gel.
- Attach the electrodes, remembering that the DNA will run towards the red (positive) electrode.
- Run the gel at ~100 volts for a few minutes.
- Look at the gel under UV light. Compare sample sizes to DNA Ladder to determine if ligation was carried out successfully.

Results:



1% agarose gel of PCR products from VF2 and VR primers

note: PD1 refers to promoter D1 (device 1 interlab study BBa_K823008), the next letter refers to who ligated the promoter to RBS+DBR2 ie. Arnas or Dáire, alpha refers to the alpha construct ie. Promoter+RBS+DBR2+RBS+ALDH1+Terminator, the next letter and number distinguish who carried out the ligation of Promoter+RBS+DBR2 and RBS

+ALDH1+Terminator and in the case of Dáire, he carried it out in duplicate so the number following D distinguishes his replicate, 1st or 2nd; the final number distinguishes which broth number the purified plasmid derives as multiple colonies were chosen from each plate.

Lane 1: DNA Ladder Lane 2: B569 PD1 A alpha D1 1
Lane 3: B569 PD1 A alpha D1 2 Lane 4: B568 PD1 A alpha D1 3
Lane 5: DNA Ladder Lane 6: B569 PD1 D alpha D1 1
Lane 7: B569 PD1 D alpha D1 2 Lane 8: B569 PD1 D alpha D1 3
Lane 9: DNA Ladder Lane 10: PD1 D alpha D2 RFP 1
Lane 11: PD1 D alpha A lin 1 Lane 12: PD1 D alpha A lin 2
Lane 13: PD1 D alpha D1 RFP 1 Lane 14: PD1 D alpha D1 RFP 2
Lane 14: DNA Ladder Lane 15: PD1 A alpha A lin 1
Lane 16: PD1 A alpha lin 2

Conclusions:
Based on the gel, the samples in Lane 10: PD1 D alpha D2 RFP 1, Lane 13: PD1 D alpha D1 RFP 1 and Lane 14: PD1 D alpha D1 RFP 2 are the expected length ~3000bp. The gel shows that the plasmid DNA in the B569 samples was not the expected length being ~1200bp not the expected ~3000bp.

Based on these results it was decided to remove the current alpha constructs from the incubator and to transform PD1 D alpha D2 RFP 1; PD1 D alpha D1 RFP 1 and PD1 D alpha D1 RFP 2 miniprepped from DH5alpha cells into B569 cells.

Transformation of B569 cells with Alpha construct 11th September:

Objective: To transform B569 cells with the alpha constructs that were the correct length based on the results of a gel.

Requirements:
- 50µL of competent cells per transformation
- purified DNA
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42^{o}C
- Ice box
- Incubator
- 950µL of SOB medium per transformation
- eppendorf tubes
- bunsen burner
- Glass spreader
- Ethanol

Procedure: (based on NEB High Efficiency Transformation protocol)

- Pre-chill eppendorf tubes on ice.

- Thaw competent cells on ice.
- Add 50µL of competent cells to each of the pre-chilled transformation tubes.
- Add 2µL of miniprepped alpha construct to its appropriate transformation tube. (.ie PD1 D alpha D2 RFP 1; PD1 D alpha D1 RFP 1 and PD1 D alpha D1 RFP 2).
- Carefully flick the transformation tubes 4-5 times to mix the cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes.
- Heat-shock at 42^{o}C in the heat-block for exactly 30 seconds.
- Place the transformations on ice for 5 minutes.
- Pipette 950µL of SOB medium into each transformation tube.
- Incubate the transformations at 37^{o}C for a few hours with shaking.
- Inoculate cultures with the transformations.

Digestion and Ligation 12th September:

Objective: To put intermediate parts and final constructs into chloramphenicol backbones for submission to iGEM HQ.

Requirements:
- NEBuffer 2.1
- Eco-RI HF®
- PstI
- SpeI
- XbaI
- dH2O
- Pipettes, pipette tips
- Water bath and/or heat block/thermocycler
- eppendorf tubes
- permanent marker
- T4 DNA ligase reaction buffer
- T4 DNA ligase
- ice box

Procedure (based on iGEM protocol http://parts.igem.org/Help:Protocols/3A_Assembly)

- Calculate the required amount of enzyme master mixes.

Prepare Enzyme master mix for plasmid backbone by adding the following to an eppendorf tube:
- 5.5µL NEBuffer 2.1
- 0.5µL Eco-RI HF®
- 0.5µL PstI
- 18.5µL dH2O

Digest RFP chloramphenicol (conc. 63.3 ng/µL) by adding the following to two labelled eppendorf tubes:
- 1.58µL RFP cm

- 2.42µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest RBS+ALDH1 D Late 1 (in kanamycin backbone) (conc. 57.5ng/µL) by adding the following to a labelled eppendorf tube:
- 1.74µL RBS+ALDH1 D Late 1
- 2.26µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest RBS+ALDH1 DL1+Terminator 1// (in ampicillin backbone) (conc. 82.3 ng/µL) by adding the following to a labelled eppendorf tube:
- 1.2µL RBS+ALDH1 DL1+Terminator 1//
- 2.8µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest Promoter D1+RBS+ALDH1+Terminator Arnas (in kanamycin backbone) (conc. 165.0 ng/µL) by adding the following to a labelled eppendorf tube:
- 0.6µL Promoter D1+RBS+ALDH1+Terminator Arnas
- 3.4µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest PTAC+RBS+ALDH1+Terminator Arnas (in kanamycin backbone) (conc. 37.1 ng/µL) by adding the following to a labelled eppendorf tube:
- 2.7µL PTAC+RBS+ALDH1+Terminator Arnas
- 1.3µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest DBR2 ST A1 (conc. 67.7 ng/µL) by adding the following to a labelled eppendorf tube:
- 1.48µL DBR2 ST A1
- 2.52µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest Promoter D1+DBR2 Dáire 1 (in ampicillin backbone) (conc. 53.1 ng/µL) by adding the following to a labelled eppendorf tube:
- 1.88µL Promoter D1+DBR2 Dáire 1
- 2.12µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest PD1 D alpha D2 RFP 1 (in kanamycin backbone) (conc. 134.6 ng/µL) by adding the following to a labelled eppendorf tube:
- 0.74µL PD1 D alpha D2 RFP 1
- 3.26µL dH2O
- 4µL enzyme master mix for plasmid backbone

Digest PD1 D alpha D1 RFP 1 (in kanamycin backbone) (conc. 19.4 ng/µL) by adding the following to a labelled eppendorf tube:
- 4µL PD1 D alpha D1 RFP 1
- 4µL enzyme master mix for plasmid backbone

Digest PD1 D alpha D1 RFP 2 (in kanamycin backbone) (conc. 22 ng/μL) by adding the following to a labelled eppendorf tube:

- 4μL PD1 D alpha D1 RFP 2
- 4μL enzyme master mix for plasmid backbone

Digest the ligations at 37^{o}C for 30 minutes. Heat-kill at 80^{o}C for 20 minutes.

Transformation 12th September: (based on NEB High Efficiency Transformation protocol)

Objective: To transform the ligation products (ie. all the intermediate constructs in chloramphenicol backbones) into DH5alpha cells.

Requirements:

- 50μL of competent cells per transformation
- purified DNA
- P20, P200 pipettes
- Pipette tips
- Heat-block set to 42^{o}C
- Ice box
- Incubator
- 950μL of SOB medium per transformation
- eppendorf tubes
- bunsen burner
- Glass spreader
- Ethanol

- Pre-chill eppendorf tubes on ice.
- Thaw competent cells on ice.
- Add 50μL of competent cells to each of the pre-chilled transformation tubes.
- Add 2μL of ligation product to its appropriate transformation tube (ie. RBS+ALDH1 D Late 1 in cm backbone; RBS+ALDH1 DL1+Terminator 1// in cm backbone; Promoter D1+RBS+ALDH1+Terminator Arnas in cm backbone; PTAC+RBS +ALDH1+Terminator Arnas in cm backbone; DBR2 ST A1 in cm backbone; Promoter D1+DBR2 Dáire 1 in cm backbone; PD1 D alpha D2 RFP 1 in cm backbone; PD1 D alpha D1 RFP 1 in cm backbone and PD1 D alpha D1 RFP 2 in cm backbone.
- Carefully flick the transformation tubes 4-5 times to mix the cells and DNA. Do not vortex.
- Place the mixture on ice for 30 minutes.
- Heat-shock at 42^{o}C in the heat-block for exactly 30 seconds.
- Place the transformations on ice for 5 minutes.
- Pipette 950μL of SOB medium into each transformation tube.
- Incubate the transformations at 37^{o}C for an hour with shaking.
- Warm selection plates to 37^{o}C (Use plates chloramphenicol for selection).
- Spin the cells at 5g for 2 minutes.
- Remove 800μL of the supernatant from each tube and discard.

- Aspirate a number of times to resuspend the pellet before plating what's left in the transformation tube.
- Pass the glass spreader through the bunsen burner's flame before spreading.
- Cool the glass spreader on part of the plate that does not contain cells before spreading the cells on the plate.
- Incubate plates overnight at 37^{o}C.

Preparation of Broths 13th September:

Objective: To make broths of parts in chloramphenicol backbones to then purify and submit to iGEM HQ.

Requirements:
- 5mL LB medium per broth
- Appropriate antibiotics
- Tubes
- Incubator with shaker
- Transformation plates
- Wire loop
- Bunsen burner
- permanent marker

Procedure:
- Add 5mL of LB medium to a falcon tube.
- Add 5µL of the appropriate 1000x antibiotic, chloramphenicol. l
- Use a sterile loop to scoop up a colony from the plate.
- Inoculate the LB medium in the falcon tube by placing the loop into the tube and stirring.
- Sterilise the loop by putting it into the bunsen burner's flame before repeating the process for the desired amount of broths.
- Incubate broths overnight with shaking at 37^{o}C.

Miniprep 14th September: (Same protocol followed as the 5th September)